University of Alberta

Synthesis and sensory evaluation of conjugated linoleic acid-enriched milk

By

John Andrew Bell

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

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in

Animal Science

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Dated: 29 May 2003

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"Synthesis and sensory evaluation of conjugated linoleic acid-enriched milk"** submitted by **John A. Bell** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Animal Science**.

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Abstract

Conjugated linoleic acid (CLA) refers to a mixture of conjugated octadecadienoic acids of predominantly ruminant origin. The main isomer in bovine milk fat is the cis-9, trans-11 18:2. Interest in CLA increased after the discovery of its health promoting properties, including potent anticarcinogenic activity. The objectives of this thesis were to evaluate dietary strategies aimed at increasing the concentration of CLA in bovine milk fat, and to evaluate the organoleptic characteristics of CLA-enriched milk.

Two feeding experiments, using a complete-randomized block design, were conducted with the aim of enriching the concentration of CLA in bovine milk. The initial study fed 28 Holstein cows for two weeks. The follow up study fed 62 Holstein cows for two months. Diets were supplemented with various combinations of safflower oil, flaxseed oil, vitamin E, and monensin. These studies showed that milk CLA concentrations could be increased by more than ten times through dietary modification.

CLA-enriched milk was processed and evaluated by a ten-person trained sensory panel and a 75-person untrained consumer panel. The trained panel found that CLA-enriched milk was not different from the control in terms of its organoleptic characteristics. Results were conflicting for consumer testing, which found one of the CLA-enriched milk samples less acceptable than control and the other CLA-enriched milk equally acceptable as control.

A final study designed as a 4x4 latin square, evaluated the effectiveness of post-ruminal delivery of a synthetic CLA mixture to increase milk CLA. Post-ruminal delivery of CLA, compared to tallow, safflower oil, or control, increased the concentration of CLA in milk fat but had extremely negative effects on milk production and composition.

These effects, which were not observed in the initial feeding studies, were attributed to the very high content of trans-10, cis-12 in the CLA mixture. The results suggested that the extent of enrichment possible for trans-10, cis-12 CLA is limited.

The results presented in this thesis demonstrated the feasibility of producing CLA-enriched milk through dietary modification. Trained sensory evaluation demonstrated that increasing the concentration of CLA did not change the organoleptic characteristics of the milk.

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List of Abbreviations

ACC = acetyl-CoA carboxylase

ADF = acid detergent fibre

ANSA = anilino-1-naphthalene sulfonic acid

BCS = body condition score

bp = base pair

°C = degrees Celcius

CCK = cholecystokinin

cDNA = complementary deoxyribonucleic acid

CHD = coronary heart disease

CLA = conjugated linoleic acid

CP = crude protein

DIM = days in milk

DM = dry matter

DMI = dry matter intake

FAS = fatty acid synthase

FCM = fat corrected milk

HSL = hormone sensitive lipase

HTST = high temperature short time

kb = kilobases

LPL = lipoprotein lipase

mRNA = messenger ribonucleic acid

NEFA = non-esterified fatty acid

NDF = neutral detergent fibre

NRC = National Research Council

OD = optical density units

PCR = polymerase chain reaction

PUFA = polyunsaturated fatty acid

rpm = revolutions per minute

RT = reverse transcription

SCC = somatic cell count

SCD =	stearoyl-CoA desaturase
SD =	standard deviation
SEM =	standard error of the mean
TG =	triglyceride
TFA =	trans fatty acids
TMR =	total mixed ration
μ =	micro

Chapter 1 – Introduction

1.1 General Introduction

Milk from ruminant animals has been an important component of the human diet for thousands of years. Ruminants have the unique ability to take organic material that is indigestible to humans and convert it into milk, a food with high nutritional value. Ancient civilizations learnt to exploit this potential through the domestication of various species of ruminants including the cow, goat, sheep, buffalo, and camel. Processing of milk into products like butter and cheese also has a long history. Cheese making is thought have been practiced as early as 4000 BC in regions of the Middle East and was a well-developed skill in the Roman era. In contrast, strategies that alter the basic composition of milk are a much more recent endeavor, encouraged by the rapid advancement of scientific knowledge over the past century.

There are many situations in which it might be advantageous to change the composition of milk. For instance, dairy farmers might be interested in changing the concentration of a particular milk component for economic reasons. In some regions where the price for milk protein is greater than for fat it has been desirable to implement feeding strategies that reduce fat concentration, increasing the protein to fat ratio. Modern dairy processors may be interested in strategies that increase casein for maximization of cheese yield, or in methods that increase unsaturated fatty acids, resulting in softer butter. Another example might be the desire of nutritionists to change milk composition in ways that promote better health. Possibilities for this include reducing the saturated fatty acid concentration or enhancing components with particular functional properties such as conjugated linoleic acid or bioactive peptides. Finally, some of the most profound possibilities for altering milk composition have come through genetic technologies. These include the use of transgenic cows designed to produce milk containing high-value pharmaceuticals of benefit to human medicine.

Although the functional and biological nature of the mammary gland undoubtedly places some constraints on the degree of change possible, it is also true that great potential exists for altering milk composition. A great deal of research has focused on the ability of nutrition to affect the percent and composition of bovine milk fat. In particular, considerable effort has been spent evaluating feeding strategies that will increase the milk fat concentration of polyunsaturated fatty acids. Interest in this area intensified following the discovery that a fatty acid found in the milk and meat of ruminants possessed anticarcinogenic properties (Pariza et al. 1985). This fatty acid was identified as a mixture of isomeric derivatives of linoleic acid with conjugated double bonds, and was henceforth named conjugated linoleic acid (CLA) (Ha, et al. 1987). This early work set off a cascade of research on the health promoting properties of CLA and on strategies to enhance the concentration of CLA in ruminant milk and meat. The focus of this thesis is the evaluation of various dietary strategies aimed at increasing the concentration CLA in bovine milk fat.

1.2 Altering the Composition of Bovine Milk through Nutrition

Nutrition offers the most effective means of rapidly altering milk composition. Although the potential for change varies according to the component, almost all the components of milk are susceptible to dietary manipulation. Fat percent and fatty acid composition is the most amenable to change whereas the content and composition of milk protein is less responsive. In contrast to milk fat, the concentration of lactose in milk is extremely resistant to change. Changes in milk composition are also not always obvious. For example, total protein concentration could remain constant while significant changes occur in the ratio of the protein fractions. Similarly, substantial changes could occur in the fatty acid composition of milk fat without alteration in milk fat percent.

1.2.1 Influence of Diet on Milk Protein

Milk from Holstein cows contains about 3.2 % (w/v) protein. This protein is made up of approximately 70-80% casein (α_{S1}, α_{S2}, β, and κ), and 20-30% whey (α-lactalbumin, β-lactoglobulin, proteose-peptones, and blood proteins). Ruminant animals have the unique ability to grow, reproduce, and lactate without a source of protein in their diets. This is possible due to the synthesis of microbial protein within the rumen from non-protein nitrogen. However, under normal circumstances the animal receives the protein it requires for milk synthesis and other functions from a combination of microbial protein and dietary protein that escapes ruminal digestion.

Increasing the level of dietary crude protein from deficient to more adequate levels (10 vs. 13 and 16% of DM) results in a linear increase in milk protein percent (Burgess and Nicholson, 1984). In addition, decreasing the rate and extent of digestion of dietary protein in the rumen has been shown to increase milk protein percent, although a greater positive effect is seen in milk yield and milk protein yield (Robinson et al., 1992; reviewed by Santos et al., 1998). Alterations in rate of degradation of dietary protein do not always increase milk protein percent, but sometimes increase non-protein nitrogen content at the expense of casein and/or whey protein (Casper and Schingoethe, 1989;

Robinson et al., 1991; Khorasani et al., 1994). Increasing the level of concentrate in the diet (above 50% of dietary dry matter) generally results in higher milk protein yield (Ingalls et al., 1980; Vinet et al., 1980). The source of starch (barley vs. corn) may also influence the characteristics of the protein fractions in milk (Khorasani et al., 1994).

Dietary supplementation of lipid often has a negative effect on milk protein percent without necessarily causing a change in protein yield (Robinson and Burgess, 1990; Khorasani et al., 1991; Erickson et al, 1992; Stegeman et al, 1992; Boila et al., 1993; Grummer et al., 1993; Drackley and Elliot, 1993; Elliot et al, 1993; Kim et al., 1993; Wu et al, 1993; Sklan et al, 1994; Wu et al, 1994). DePeters, et al. (1992) demonstrated that this decrease in percentage protein could not be entirely due to the dilution effect of increased milk yield.

1.2.2 Influence of Diet on Milk Lactose

Lactose, a disaccharide of glucose and galactose, is the predominant carbohydrate in milk making up 4.6% w/v of Holstein milk. The glucose that the mammary gland uses to synthesize lactose is derived from liver gluconeogenesis (to a large extent from propionic acid) and from glucose absorbed from the small intestine. Lactose is the most important osmotic constituent in milk. Therefore, any increase in lactose synthesis is followed by an increase in water secretion and hence increased milk volume. Conversely, if lactose synthesis is reduced, milk volume declines. Attempts to influence milk yield by infusions of propionate and/or glucose has yielded disappointing results (Hurtaud et al., 1993). A significant proportion (minimum 20%) of dietary starch from cereal grains escapes digestion in the rumen (Robinson et al., 1994). This starch releases significant quantities of glucose to the small intestine, which may suggest that milk production is less limited by supply of lactose precursors than was traditionally thought (Okine and Kennelly, 1994).

1.2.3 Synthesis of Ruminant Milk Fat

The concentration of fat in Holstein milk is generally in the range of about 3 to 4.5% w/v. Milk fat is a complex lipid containing more than 400 distinct fatty acids. Most of these fatty acids are esterified to glycerol as triglycerides, which make up 97 to 98% of the milk lipid. The remainder of the fat is mainly comprised of much smaller amounts of phospholipids, cholesterol, cholesterol esters, diglycerides, monoglycerides, and free fatty acids. The prominent features of bovine milk fat include the presence of short- chain fatty acids, the presence of odd and branch-chain fatty acids, a relatively high proportion of saturated fatty acids, a low proportion of polyunsaturated fatty acids, and the presence of various trans fatty acids, including conjugated linoleic acid (CLA).

Figure 1.1 gives an overview of the synthesis and secretion of milk fat in the ruminant mammary gland. The fatty acids in bovine milk originate either from preformed fatty acids, coming from the rumen or mobilization of body fat stores (ca. 60%), or through de-novo synthesis from acetate and β-hydroxybutyrate (ca. 40%) (Bauman and Davis, 1974; Baber et al., 1997).

Rumen fatty acids originate either from the diet or microbial synthesis. The odd chain and branched chain fatty acids in bovine milk come from microbial synthesis. The biohydrogenation of dietary unsaturated fatty acids by rumen microbes produces trans fatty acids and is responsible to a large extent for the high proportion of saturated fatty acids in bovine milk fat. Dietary and microbial fatty acids flow to the small intestine where they are re-esterified and packaged into chylomicrons in the intestinal enterocyte. From here they are taken up by the mesenteric lymph, and released into the bloodstream. At the mammary gland (and other tissues) the chylomicrons undergo hydrolysis on the endothelial surface of blood capillaries through the action of lipoprotein lipase (LPL).

The other source of preformed fatty acids for milk fat synthesis is the mobilization of adipose tissue. These fatty acids are hydrolyzed from adipose through the action of hormone-sensitive lipase (HSL) and travel to the mammary gland as non-esterified fatty acids (NEFA) bound to albumin. The amount of fatty acids coming from fat stores depends on the physiological state of the animal. Fat mobilization occurs to the greatest extent during early lactation when the cow tends to be in a state of negative energy balance.

De-novo synthesis of fatty acids occurs in the cytosol of mammary epithelial cells and involves two key enzymes, acetyl-CoA carboxylase and fatty acid synthase (Chilliard et al., 2000). Acetyl-CoA carboxylase (ACC) catalyses the formation of the three-carbon malonyl-CoA from the two-carbon acetate. Fatty acid synthase (FAS) catalyses condensation cycles of malonyl-CoA with either acetyl-CoA or the four-carbon butyryl-CoA. De-novo synthesis of fatty acids produces most of the short and medium chain saturated fatty acids from 4:0 to 14:0, and approximately half the 16:0.

Unlike other ruminant tissues, the lactating mammary gland is unable to convert 16:0 to 18:0 by chain elongation (Moore and Christie, 1981). The ruminant mammary gland does, however, possess stearoyl-CoA desaturase (SCD), which converts a substantial amount of the blood derived 18:0 to 18:1 n-9 (Kinsella, 1972). The desaturase system is a multienzyme complex that includes NADH-cytochrome b_5 reductase, cytochrome b_5, acyl CoA synthase, and the terminal Δ-9 desaturase (Ntambi, 1999). Stearoyl-CoA and palmitoyl-CoA are the main substrates for the Δ-9 desaturase reaction, which introduces a *cis*-double bond between carbons 9 and 10 on the fatty acid. The formation of these monounsaturated fatty acids is important for the maintenance of membrane fluidity.

Esterification of fatty acids into triglycerides occurs in the endoplasmic reticulum. From here the triglycerides are directed to the cytoplasmic side of the membrane where they collect as lipid droplets. These droplets move to the apical plasma membrane and are expelled into the alveolar lumen by pinocytosis, picking up a coating of plasma membrane in the process.

1.2.4 Influence of Diet on Milk Fat

As stated above, milk fat is the most variable of the milk components in terms of amount and composition. The factors associated with this variation include genetics, stage of lactation, nutrition and other factors influencing rumen fermentation characteristics (Jensen, 2002). Researchers have been interested in modifying the composition of milk fat for decades. Much of this work has focused on feeding various sources of dietary lipid for the purpose of increasing the level of particular fatty acids: most often n-3 and n-6 polyunsaturated fatty acids. More recently, particular emphasis has been given to finding feeding regimens that will increase the concentration of CLA, a powerful anticarcinogen found naturally in ruminant milk fat. Despite the large volume of knowledge that has accumulated on how to modify the composition of cow's milk, very little of this information has been applied in a commercial setting. Yet, the ability to modify the composition of milk fat represents an opportunity for the development of novel dairy products such as CLA-enriched milk.

The influence of nutrition on bovine milk fat has been reviewed a number of times in recent years (Kennelly, 1996; Ashes, et al, 1997; Mansbridge and Blake, 1997; Bauman and Griinari, 2001; Chilliard, et al., 2001b; Jensen, 2002). The following sections will provide an overview of the principal nutritional factors that affect milk fat concentration and composition, followed by a more detailed survey of the effect of nutrition on milk CLA.

1.2.4.1 Influence of Diet on Milk Fat Percent

Increasing the fat concentration of milk has traditionally been an important goal of dairy breeding programs. This reflected the market demand for milk fat and a price incentive that rewarded higher fat concentration. In recent years there has been a decline in the demand for milk fat as consumption patterns changed. This could be seen in the substitution of margarine for butter, and a consumer preference for reduced-fat dairy products. Per capita consumption of 3.25% milk declined in Canada every year between 1980 and 2001 so that by 2001 over 80% of total milk consumption was in the form of reduced-fat milk (Statistics Canada, 2001). Modification of the dairy diet to influence milk fat concentration could be extremely useful for better matching milk fat production with demand.

The earliest citing of diet causing a depression in milk fat percent was 1845 in a study where beets were fed to dairy cows (reviewed by Bauman and Griinari, 2001). In the first half of the twentieth century milk fat depression was observed with a range of dietary ingredients including, cod liver oil (Drummand et al., 1924; Golding et al., 1926), diets low in roughage/high in concentrate (Powell, 1939; Loosli et al., 1945; Balch et al., 1952), and diets containing supplementary plant oils (Dann et al., 1935; Williams et al., 1939).

Increasing the proportion of concentrate in the diet causes a depression in milk fat percent with the effect being particularly pronounced as the concentrate exceeds 50 to 60% of dietary dry matter (Ingalls et al., 1980; Vinet et al., 1980; Steacy et al, 1983). Kennelly and Glimm (1998) presented similar findings when comparing concentrate levels of 50 vs. 75% of dietary DM at early and late lactation. These studies showed a decline in milk fat percent going from 50 to 75% concentrate, with the effect being more pronounced in late lactation. Earlier research attributed this decrease in milk fat to a decrease in rumen production of acetate (Van Soest, 1963). Increasing the proportion of grain in the diet was shown to decrease the molar ratio of acetate to propionate and there appeared to be a clear relationship between these changes in rumen volatile fatty acids and milk fat. However, it was later shown that the reduction in the proportion of acetate was actually due to an increase in propionate rather than a reduction in acetate and researchers concluded that a deficiency in acetate could not account for the observed reduction in milk fat (Davis and Brown, 1970).

Another theory, called the glucogenic-insulin theory, was based on the hypothesis that the increased propionate would increase insulin levels and result in a channeling of nutrients to the adipose and away from the mammary gland (Jenny et al., 1974). Adipose tissue, unlike bovine mammary tissue, is sensitive to increases in insulin levels and responds with increased rates of lipogenesis and decreased levels of lipolysis. It was proposed that the increase in insulin would cause an increase in adipose use of acetate, β-hydroxybutyrate, and diet-derived long chain fatty acids, as well as a reduction in mobilization of fatty acids from body reserves. This would cause a shortage of nutrients required for milk fat synthesis explaining the depression in milk fat. Convincing evidence against this theory was provided by studies that employed a chronic hyperinsulemic-euglycemic clamp procedure (Griinari et al., 1997b). The procedure provided a means to increase blood insulin levels while avoiding hypoglycemia, which could have caused counter-regulatory changes in glucose homeostasis (Bauman and Griinari, 2001). Using this method, Griinari et al. (1997b) found minimal effects on milk fat synthesis with elevated insulin. Furthermore, other studies reported situations where changes in feeding elevated insulin but had no effect on milk fat (Griinari et al, 1998).

Feeding diets supplemented with highly polyunsaturated oils (e.g., vegetable oils and fish oils) have also been widely documented as causing a depression in milk fat percent (Bauman and Griinari, 2001). In contrast, abomasal infusion of oils, or feeding rumen-protected oil, often increases milk fat percentage (Schauff, et al. 1992; Sklan, 1994; Wu, et al. 1994). One theory attributed this difference to a decrease in fiber digestion with oil feeding (Byers and Schelling, 1988; Jenkins, 1993), caused either as a result of a coating effect of the oil on the forage, or by a direct toxic effect of polyunsaturated fatty acids on rumen microbes (Palmquist, 1988). It was proposed that a decrease in fiber digestion would lead to a decrease in rumen acetate, limiting the availability of milk fat precursors. Another theory proposed that trans-18:1 fatty acids might be a cause of this decrease in fat after it was observed that these fatty acids were increased in milk during milk fat depression (Davis and Brown, 1970; Emery, 1973; Pennington and Davis, 1975). The rumen outflow of trans 18:1 was found to be significantly greater with diets causing milk fat depression (Wonsil et al., 1994; Kalscheur, et al. 1997). Further evidence was provided in studies that abomasally infused trans 18:1 fatty acids where a depression in milk fat percent of between 14 to 25% was reported (Selner and Schultz, 1980; Gayner, et al. 1994; Romo et al. 1996). More recent work has shown that this depression is caused more specifically by trans-10 18:1 and trans-10, cis-12 18:2 formed during the biohydrogenation of polyunsaturated fatty acids in the rumen (Figure 1.2) (Griinari et al, 1997, 1998). In fact, a strong curvilinear relationship (R^2=0.99) has been reported between yield of milk fat and dose of trans-10, cis-12 CLA infused abomasally (Peterson et al., 2002). Griinari, et al. (1998) also showed that high concentrate diets could alter the products of rumen biohydrogenation of polyunsaturated fatty acids resulting in an increase in the proportion of trans-10 18:1 and trans-10, *cis*-12 CLA isomers. This theory, coined the biohydrogenation theory, has been useful in explaining milk fat depression under various feeding situations such as high grain diets or lipid supplementation.

1.2.4.2 Influence of Diet on Milk Fat Composition

The main nutritional factors affecting milk fat composition include the effect of forages, rumen modifiers, and supplemental fats and oils. These factors can influence the milk fatty acid composition by providing dietary preformed fatty acids, by influencing the rumen production of precursors for de-novo synthesis, by affecting rumen microbial fatty acid synthesis, and through the rumen production of specific fatty acids that either inhibit or stimulate de-novo synthesis.

Forages have an influence on milk fatty acid composition in two ways. Firstly, forage provides much of the substrate for cellulytic bacteria, the main producers of acetate and butyrate in the rumen. Secondly, forages can contribute a significant amount to the total fatty acid intake (Mansbridge and Blake, 1997), primarily in the form of polyunsaturated fatty acids. Fresh grass in temperate countries contains 1-3% fatty acids with α-linolenic acid (18:3 n-3) making up as much as 55-65% of the fatty acids (Bauchart et al., 1984). Milk produced from cows fed diets based on grass or grain silage (over 60% DM) was reported to have a higher proportion of 14:0 and 16:0 and a lower proportion of 18:1, 18:2 and 18:3 compared to pasture fed cows (Chilliard et al., 2001b).

Dietary additives that modify the rumen conditions can also have an impact on the milk fatty acid profile. Kennelly et al. (1999) evaluated the effect of the addition of buffer to diets containing 50:50 or 25:75 forage:concentrate. Cows fed the high-concentrate diet had lower ruminal pH, lower ruminal acetate and butyrate concentrations, and higher propionate concentrations compared to the medium concentrate diet. The addition of buffer resulted in elevated total volatile fatty acid concentrations and acetate concentration. Moreover, the addition of buffer prevented the elevation in milk trans 18:1 and the associated depression in milk fat percentage. Monensin has also been shown to affect the amount and composition of milk fat (Sauer et al., 1998). Monensin is an ionophore antibiotic that acts by altering the movement of ions across the membranes of gram-positive bacteria (Schelling, 1984). Gram positive bacteria tend to be the acetate and hydrogen formers in the rumen whereas gram negative bacteria, which are resistant to ionophores, produce propionate. The use of ionophores tends to increase the propionate to acetate ratio and reduces proteolysis and methane production in ruminants. For these reasons they are widely used in beef feed-lot operations because they improve production efficiency. Some of the reported effects of adding ionophores to the dairy diet include improved energy metabolism, a reduction in the incidence of ketosis, increased milk production, and small decreases in milk protein and fat content (Jensen, 2002). Ionophores have been shown to decrease the rumen biohydrogenation of polyunsaturated fatty acids in vitro (Fellner et al., 1997). This was supported by in vivo data of Sauer et al. (1998) who found an increase in milk cis-18:1, trans-18:1, 18:2 and CLA and a decrease in 10:0, 12:0, 16:0, and 18:0 with the addition of 24 ppm monensin to the diet.

The addition of supplemental fats and oils to the diet has the greatest influence on the amount and composition of bovine milk fat. Some of the common supplemental fats and oils that have been tested include whole and processed oilseeds or the extracted oil, animal fats, marine oils, and rumen-protected fats. A great deal of work has been done on the effect of feeding oilseeds or the oil from oilseeds, to dairy cows (Kennelly, 1996). The typical composition of a variety of oilseeds is shown in Table 1.1. In general, feeding processed oilseeds or oils like canola, soybean, sunflower, safflower, or flaxseed results in a reduction in milk 4:0 - 16:0 and an increase in 18:1, 18:2, or 18:3; the extent of change in fat composition will depend on the amount and type of oilseed supplemented (Sutten, 1989; Drackley et al, 1992; Kennelly and Khorasani, 1992, Delbecchi et al., 2001). However, the degree of enrichment for these mono- and polyunsaturated fatty acids is much lower than might be predicted from the fatty acid composition of the oilseed. The reason for this lies in the ability of the rumen microbes to effectively hydrogenate unsaturated fatty acids. Researchers have attempted to get around this problem by protecting the oil from microbial biohydrogenation, most often by encapsulating the lipid in formaldehyde-treated casein or by feeding the fatty acids as calcium salts. The extent of protection obtained with these methods can be variable (Chilliard et al., 2001b). Animal fats such as beef tallow have been used as an effective means of increasing the energy density of the diet (Mansbridge and Blake, 1997). The major fatty acids in beef tallow are 16:0 (23-27%), 18:0 (14-29%), and 18:1 (36-50%) (Chilliard et al., 2001b). Supplementing beef tallow tends to decrease 6:0-14:0 and increase 18:1 in milk fat (Weigel et al, 1997; Chilliard et al., 2001b). Because of its low content of polyunsaturated fatty acids, beef tallow likely has limited value for the specific purpose of increasing the level of unsaturated fat in milk. Furthermore, it also suffers from the negative image surrounding the feeding of animal products to animals. A great deal of interest has been shown in the potential to modify milk fat composition through supplementation of marine oils. Marine oils tend to be rich in the very long chain polyunsaturated fatty acids, 20:5 and 22:6, although the actual amounts and proportions of these fatty acids vary widely from one fish species to another. Fish oil feeding generally tends to decrease the proportion of 4:0–14:0, 16:0, 18:0, and 18:1 and increase the trans 18:1, 20:5 and 22:6 in milk (Chilliard et al., 2001b). The increase in 20:5 and 22:6 is generally fairly small as transfer efficiencies for 20:5 and 22:6 appear to be low (Offer et al., 1999).

The discovery of the anticarcinogenic properties of CLA presented exciting new possibilities for the production of dairy products with modified fat content. Researchers quickly began to explore ways to increase this fatty acid in bovine milk through manipulation of the dairy diet. The interesting aspect that makes increasing CLA in milk so different from increasing other types of unsaturated fatty acids is that while previous work struggled with the problem of avoiding the effects of rumen biohydrogenation, increasing CLA is dependent on the biohydrogenation process.

1.3 Conjugated Linoleic Acid

The topic of CLA as it relates to ruminant production has been reviewed previously (Griinari and Bauman, 1999; Dhiman, 2000; Chilliard, et al., 2001b). Conjugated linoleic acid (CLA) is a component of milk fat that has been shown in recent years to have numerous potential benefits for human health, including potent cancer-fighting properties. This is especially interesting considering that most naturally occurring anti-carcinogens are of plant origin. Since CLA is a product of ruminant animals, bovine milk and milk products are among the richest dietary sources.

1.3.1 Biosynthesis of CLA in the Cow

A summary of CLA synthesis in the cow is shown in Figure 1.2. The presence of CLA in products of ruminant origin can be attributed to the process of rumen biohydrogenation. This process, carried out by rumen bacteria, ultimately serves to remove double bonds from unsaturated fatty acids and is the reason for the high levels of saturated fatty acids found in the meat and milk of ruminants. Intermediate products in the biohydrogenation process include isomers of CLA and trans-18:1. The CLA found in ruminant meat and milk appears to originate either directly from the rumen or by tissue desaturation of rumen-derived trans-11 18:1.

1.3.1.1 Formation of CLA in the Rumen

The forages and grains fed to dairy cows are characterized by a relatively high content of linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acid. Prior to rumen biohydrogenation of these polyunsaturated fatty acids, dietary lipids undergo hydrolysis of ester bonds yielding unesterified fatty acids and glycerol. In fact, hydrolysis is a prerequisite for biohydrogenation because of the requirement for a free carboxyl group. The whole process occurs mostly through the action of bacterial extracellular enzymes, with protozoa playing only a minor role (Harfoot and Hazelwood, 1988).

The biohydrogenation process involves several steps and complete biohydrogenation requires the participation of more than one species of bacteria (Kemp and Lander, 1984). A range of rumen bacteria have been shown to have the ability to hydrogenate unsaturated fatty acids (Harfoot and Hazelwood, 1988; Fujimoto et al., 1993) (Figure 1.3). Kepler et al (1966) showed that cis-9, trans-11 18:2, the major isomer of CLA, is the first intermediate formed in the biohydrogenation of linoleic acid by the rumen bacteria, ***butyrivibrio fibrisolvins.*** This initial reaction involves the isomerization of the *cis*-12 double bond to trans-11 by Δ-12 cis, Δ-11 trans isomerase (Kepler and Tove, 1967; Kepler et al., 1970). The enzyme binds to the 18:2 n-6 through interaction of its active site with the π-electrons on carbon-9, and by hydrogen bonding between the electronegative region of the enzyme and the fatty acid carboxyl group. A hydrogen atom is transferred from carbon-11 to the carboxyl group followed by the transfer of a hydrogen atom from the enzyme to carbon-13 producing cis-9, trans-11 18:2.

The next step is the reduction of this diene to a trans-11 monoene (trans-11 18:1). These initial steps occur rapidly suggesting that there is little accumulation of CLA in the rumen. The conversion of trans-11 18:1 to 18:0 appears to involve a different group of organisms and occurs at a slower rate (Harfoot et al 1973b; Kellens et al, 1986; Griinari et al. 1997a). For this reason, trans-11 18:1 typically accumulates in the rumen. Trans-11 18:1 and cis-9, trans-11 18:2 account for approximately 50% of the trans fatty acids found in milk fat (Griinari, 1998). Although the cis-9, trans-11 is the predominant CLA isomer in bovine milk (Parodi, 1977), other isomers can be formed with double bonds in positions 7/9, 8/10, 9/11, 10/12, or 11/13 (Sehat et al., 1998). Each of these double bonds can be in a cis or trans configuration, giving a range of possible CLA isomers. The term conjugated linoleic acid refers to this whole group of 18 carbon conjugated fatty acids. Alpha-linolenic acid goes through a similar biohydrogenation process producing 18:1 trans-11 and 18:0, but does not appear to produce CLA as an intermediate. It has been estimated that as much as 80% and 92% respectively of linoleic and linolenic acids disappear from the rumen via biohydrogenation (Doreau and Ferlay, 1994).

Oleic acid (18:1 cis-9) is another typical fatty acid found in ruminant feeds. This fatty acid is mostly hydrogenated to stearic acid and is not a precursor for CLA in the rumen. However, recent evidence has shown that oleic acid can also be converted to *trans* 18:1 isomers by rumen bacteria (Mosely, et al 2001).

1.3.1.2 Synthesis of CLA in Ruminant Tissues

Although it is accepted that CLA is formed in the rumen, there is strong evidence that much of the cis-9, trans-11 CLA found in bovine milk is actually synthesized within the mammary gland from trans-11 18:1 (Griinari and Bauman, 1999). This is possible through the action of stearoyl-CoA desaturase (SCD), an enzyme capable of adding a cis-

9 double bond to trans-11 18:1 to give cis-9, trans-11 18:2 (Mahfouz et al., 1980; Pollard et al., 1980). Stearoyl-CoA desaturase expression has been shown in ruminant adipose (Chang et al., 1992; Cameron et al., 1994; Page et al., 1997), and ruminant mammary tissue (Bickerstaffe and Annison, 1970; Kinsella, 1972). The mammary gland is the major site of Δ-9 desaturation in the lactating ruminant (Griinari et al., 2000).

A close relationship between milk cis-9, trans-11 18:2 and trans-11 18:1 has been found in numerous studies (Jiang et al., 1996; Jahreis et al., 1997; Precht and Molkentin, 1997; Griinari and Bauman, 1999; Peterson et al., 2002), consistent with a product-precursor relationship. Abomasal infusion of trans-11 18:1 (12.5 g/d) was found to increase the concentration of CLA in milk fat by 40% (Corl et al., 1998). Adding to this, Corl, et al. (2000) showed that the level of cis-9, trans-11 in milk was reduced by over 60% after abomasal infusion of sterculic oil, a potent inhibitor of Δ^9-desaturase. These studies provided strong evidence that endogenous synthesis via SCD is the major source of CLA in bovine milk fat.

1.3.2 Conjugated Linoleic Acid content of Foods and Estimated Intake

The CLA content of various foods is shown in Table 1.2. Milk and meat from ruminants contains more CLA than that of non-ruminants (Chin et al, 1992; Fritsche and Steinhart, 1998). The amount of CLA found in dairy fat is generally about 3 to 6 mg/g fat (Lin et al., 1995; Ma et al., 1999), although some studies have reported much wider variation (Kelly and Bauman, 1996; Lavillonnière et al., 1998). A large part of the variation in milk CLA can be explained by diet (discussed in 1.3.4) although other factors such as breed, parity, and stage of lactation likely play a role. White et al (2001) found that Holstein cows tended to have a higher concentration of CLA in their milk than Jersey cows. In another study, milk from Brown Swiss cows was reported to contain more CLA than Holstein milk, although Brown Swiss milk appeared to be less responsive to dietary manipulation (Whitlock, et al., 2001). Variation in Δ^9-desaturase may explain much of this difference between breeds. Age of the cow and stage of lactation may influence the milk CLA content to some degree but the effect of these parameters has not been well characterized. Processing and storage of the milk appear to have minimal effects on milk CLA concentration (Shantha et al., 1992; Shantha et al., 1995). The concentration of CLA in food therefore depends to the greatest extent on the concentration in the raw material.

Although intake of CLA varies widely between individuals and from country to country, most estimates place average intake at less than 1g/d. Intake of CLA in North America has been estimated at 52 to 137mg CLA/day (Ritzenenthaler et al 1998). This is similar to a survey of young Canadian adults where intake was 15 to 174 mg/d (average 94.9 ± 40.6) (Ens et al., 2001). Slightly higher intakes where estimated in a German survey where intake was 430 mg/d and 350 mg/d for men and women respectively.

The fact that ruminant milk contains CLA has been known for a long time. The unexpected effects of these fatty acids on health have only been discovered in more recent years.

1.3.3 Potential Health Benefits of CLA

Milk fat contains many substances that may have beneficial effects on human health (Parodi, 1997, Molkentin, 1999). Epidemiological data has given some support for an inverse association between intake of dairy products and breast cancer occurrence, although the evidence is far from unequivocal (Knekt and Järvinen, 1999). A Finnish national public health study covering a 25-year period indicated that as the intake of dairy products increased, the risk of breast cancer decreased (Knekt et al., 1996). This strongly suggested that dairy products contained some component or components capable of affording significant benefits to human health.

Most substances in nature that demonstrate anti-carcinogenic activity are of plant origin and are only present at trace levels (Wattenberg, 1992). In contrast, CLA is found almost exclusively in animal products and has been shown to be one of the most potent of all naturally occurring anti-carcinogens. The origins of research in this area can be traced to studies from the laboratory of Michael Pariza at the University of Wisconsin. While studying the effects of temperature and time on mutagen formation in pan-fried hamburger they obtained evidence for mutagenic inhibitory activity in the uncooked and fried hamburger (Pariza et al. 1979). Pariza and Hargreaves (1985) subsequently partially purified the mutagenesis inhibitor from fried ground beef and showed that it was capable of inhibiting the initiation of chemically induced mouse epidermal tumors. This was the first study to show that ground beef contained an anti-carcinogen that was effective in an intact animal. That cooked beef contained a substance that could inhibit tumor growth was intriguing since it was well known that the cooking of protein-rich foods could produce a range of mutagens and carcinogens. The next stage of the research was to elucidate the identity of the anti-carcinogen. Pariza and Hargreaves (1985) had earlier noted that the anticarcinogen was a nonpolar molecule. Subsequent work from Pariza's laboratory showed that it was actually a mixture of four isomeric derivatives of linoleic acid, each containing a conjugated double-bond system (Ha, et al. 1987). The anticarcinogenic mixture was henceforth designated as conjugated linoleic acid or CLA. To prove that the anticarcinogenic effects were indeed due to CLA they tested a synthetically prepared mixture of the CLA isomers on the mouse tumor model. The CLA-treated mice developed only about half as many papillomas and exhibited a lower tumor incidence compared with the control mice (Ha, et al. 1987). This initial work started a cascade of research. CLA has since been shown to demonstrate a range of biological effects including anticarcinogenic, antiatherogenic, antiadipogenic, and antidiabetic effects. Most studies to date have used synthetic mixtures of CLA isomers composed mostly of 8/10, 9/11, 10/12, and 11/13 isomers. Unless otherwise stated, the *in vivo* studies mentioned in sections 1.3.3.1 to 1.3.3.5 used this synthetic CLA mixture.

1.3.3.1 Anticarcinogenic Effects of CLA

Conjugated linoleic acid has since been shown to be effective in experimental animal models of mouse skin carcinogenesis, mouse forestomach tumorigenesis, and rat mammary tumorigenesis (Belury, 1995). It was effective *in vitro* with breast tumor cells, malignant melanoma, colorectal cancer cells, leukemia, prostate carcinoma and ovarian carcinoma (Scimeca, 1999). It seems to act in a dose dependent manner as demonstrated *in vitro* with breast cancer cells (Shultz, et al 1992) and *in vivo* with chemically induced mammary tumors in rats (Ip et al. 1994). Feeding as little as 0.05g CLA/100g of diet caused a reduction in the number of mammary tumors (Ip, et al 1994). Ip et al (1999) evaluated the effect of CLA enriched butter on mammary tumors in rats. The butter contained 4.1% CLA, 92% of which was the cis-9, trans-11 isomer. CLA enrichment was achieved by including sunflower oil in the diet of 20 dairy cows at 5.3% of dry matter. Milk was collected from nine of these cows that were producing the highest levels of CLA in their milk. They showed that CLA enriched butter was able to inhibit rat mammary tumor yield by 53%. This study clearly showed that the predominant isomer in ruminant products, the cis-9, trans-11 isomer, was anti-carcinogenic.

1.3.3.2 Antiathergenic Effects of CLA

There is some evidence that dietary CLA decreases atherosclerotic plaque formation. A study feeding rabbits a hypercholesterolemic diet, with or without CLA at 0.5 g/d, found that the diets containing CLA reduced serum triglycerides and low density lipoprotein cholesterol levels, and reduced atherosclerotic plaque formation, compared to diets without CLA (Lee et al., 1994). Similar results were obtained in hamsters. Diets including CLA at 1.0% w/w of the diet reduced total plasma cholesterol, non-high density lipoprotein-cholesterol, and early aortic atherosclerosis (Nicolosi et al. 1997). Looking at the effects of CLA on the thrombotic properties of blood cells, CLA, cis-9, trans-11, and trans-10, cis-12 were found to inhibit collagen or arachidonate-induced platelet aggregation in cultured platelets (Truitt et al., 1999). However, in a human study, supplementation of 3.9 g/d of a CLA mixture compared to a sunflower oil placebo, did not produce any differences in platelet aggregation or prothrombin time (Benito et al., 2001). Furthermore, in contrast to the encouraging effects observed in studies with rabbits and hamsters, CLA was shown to promote fatty streak formation in C57B1/6 mice (Munday et al., 1999). Further work will be necessary to resolve these apparent differences to determine if CLA could have a role in reducing cardiovascular disease in humans.

1.3.3.3 Antiadipogenic Effects of CLA

Numerous studies have demonstrated the ability of CLA to reduce the accumulation of adipose tissue in various species including mice, rats, pigs, and humans (Dugan et al., 1997, Park et al., 1997; Ostrowska et al., 1999; Sisk et al., 2001; Smedman and Vessby, 2001). The effect appeared to be isomer specific with the trans-10, cis-12 being more effective than the cis-9, trans-11 isomer (Park et al., 1999). In some studies the changes in adipose mass were quite dramatic. Park et al. (1997) reported a 57 to 60% reduction in lower body fat and a 5 to 14% increase in lean body mass in mice relative to controls. Many of these studies showed the additional benefit of improved feed conversion efficiency (Dugan et al., 1997, 1999; Ostrowska et al., 1999). The ability of CLA to repartition nutrients from fat towards lean and increase feed efficiency in meat-producing animals could have huge economic benefits (Dugan et al., 1997). In adult humans the ability of CLA to reduce adipose tissue mass has been less certain with some studies reporting an effect (Blankson et al., 2000; Smedman et al., 2001; Thom et al., 2001) while others failed to show any benefit (Zambell et al., 2000; Mougios et al., 2001). Differences in dose, isomeric composition of CLA used, study duration, or differences in initial body composition of subjects, may have contributed to the observed differences.

1.3.3.4 Antidiabetic Effects of CLA

Type 2 diabetes mellitus is a disease that affects about two million Canadians. The characteristic feature of the disease is an insulin resistance that results in elevated postprandial blood glucose levels. Obesity appears to be a primary risk factor (Diabetes

Prevention Program Research Group, 1999). Studies from the laboratory of Martha Belury were the first to show that CLA might have benefits for the treatment of diabetes. Zucker *fa/fa* diabetic rats were fed a diet containing 1.5% w/w CLA for two weeks. Compared to a control group, rats fed the CLA diet exhibited significantly reduced fasting blood glucose, insulin, triglycerides, and free fatty acids (Houseknecht et al. 1998; Belury and Vanden Heuvel, 1999). As in the effect of CLA on adipose accumulation, the effect of CLA on diabetes appears to be specific to the trans-10, cis-12 isomer (Ryder et al., 2001). A study looking at the ability of dietary CLA to aid in the management of type 2 diabetes in humans has yielded encouraging results (Belury, 2002). CLA supplementation (6.0 g CLA/d) was carried out for eight weeks on subjects with type 2 diabetes in a double blind, randomized study. Subjects receiving CLA supplementation had significantly lower fasting blood glucose, plasma leptin, body mass index, and weight.

1.3.3.5 Other Effects of CLA

A remarkably broad range of health benefits has been suggested for CLA. Apart from the aforementioned effects on carcinogenesis, atherosclerosis, obesity, and diabetes, CLA may have a role in counteracting immune induced muscle wasting in poultry (Cook et al. 1993) and have beneficial effects on bone formation (Watkins et al. 1999).

As mentioned above, most of the studies carried out so far have used a mixture of CLA isomers. These mixtures are composed mainly of the cis-9, trans-11 and trans-10, cis-12 isomers but a range of other isomers are also present. As research continues, the specific physiological effects of each of the isomers will be better defined. However, as shown above, there is good evidence that the predominant isomer in milk fat, the cis-9, trans-11, possesses potent anticarcinogenic activity.

1.3.4 Metabolism of CLA

Conjugated linoleic acid fed in the diet of animals and humans is known to accumulate in various tissues (Yurawecz et al., 1999). However, the cis-9, trans-11 isomer has been shown to accumulate to a greater extent than the trans-10, cis-12 in both tissue phospholipids (Banni et al., 2001) and neutral lipids (Ip et al., 1999; Eggert et al., 2002). Reasons for this could be a preference for incorporation of cic-9, trans-11 and/or a preference for more rapid metabolism of the trans-10, cis-12.

Isomers of CLA are substrates for the same Δ6-desaturation – elongation - Δ5-desaturation pathway as linoleic acid, resulting in conjugated 18:3, conjugated 20:3, and conjugated 20:4, respectively. In an enzymatic assay using a hepatic isolate of Δ6-desaturase, Belury and Kempa-Steczko (1997) showed that CLA and linoleic acid were metabolized to the same extent. Sébédio et al. (1997) also demonstrated that CLA is subject to peroxisomal β-oxidation. Like the CLA isomers themselves, metabolites of CLA are readily incorporated into phospholipid and neutral lipid fractions of various tissues (Belury, 2002). The potential biological activity of these various CLA metabolites requires further investigation.

The mechanism, or mechanisms, through which CLA isomers act has been the subject of much speculation. One mechanism that has received a great deal of attention is the effect of CLA on arachidonate-derived eicosanoids. The ability of CLA to reduce cyclooxygenase products like PGE_2 and $PGF_{2\alpha}$ has been demonstrated in vivo in bone and macrophages (Li and Watkins, 1998; Sugano et al., 1998). The effect of CLA on eicosanoids may be the result of CLA displacing arachidonate in phospholipids, or possibly through CLA inhibition of cyclooxygenase-1 or cyclooxygenase-2 at the level of mRNA, protein, or activity (Belury, 2002). Altering eicosanoid intracellular signaling could effect a range of biological activities including lipid metabolism and cytokine synthesis/function (Pariza t al., 2000). The ability of CLA to act through eicosanoids or other potential mechanisms is an active and evolving area of research.

1.3.5 Increasing the Concentration of CLA in Milk

In view of the potential benefits of CLA for human health, a number of researchers began looking at possible ways of increasing the concentration of CLA in bovine milk fat. There appears to be two practical approaches to achieve this goal. The first approach is to use dietary modification in an attempt to increase the natural production of CLA in the cow. The second approach is to feed the synthetic mixture of CLA isomers, protected in some way from the microbial biohydrogenation in the rumen. Both approaches will be discussed below.

1.3.5.1 Increasing Milk CLA through Dietary Manipulation

The concentration of CLA in bovine milk fat can vary quite substantially depending on the feeding strategy adopted. For instance, pasture feeding has been found to result in a much higher milk fat CLA concentration than that achieved with typical total mixed rations (TMR) based on conserved forage and grain (Kelly et al, 1998b;

Dhiman, et al., 1999; White, et al., 2001). Dhiman, et al. (1999) reported the CLA concentration of milk to be 22.1mg/g fat with pasture feeding compared to 3.8mg/g fat with TMR feeding. Although the lipids in pasture forages are in the form of glycolipids and phospholipids, they are hydrolyzed and hydrogenated in the same manner as triglycerides (Dawson et al, 1974; Singh and Hawke, 1979). However, one important difference between feeding pasture verses conserved forage and grain is the higher percentage of 18:3 n-3 (as much as 55-65% of fatty acids) in pasture (Bauchart et al., 1984). Furthermore, the overall diet composition and feeding characteristics of pasture are quite different to typical TMR feeding based on conserved forage and grain. For instance, the source and content of dietary carbohydrate may influence microbial fermentation in ways that alter the production of rumen CLA and trans 18:1. Other factors might include differences in passage rate and fluid dilution rate due to the high water intake associated with grazing pasture, differences in meal size, feeding frequency, bite size, or in time spent ruminating (Kelly et al., 1998b). Forage maturity also appeared to be a factor in one study where early growth stage pasture resulted in higher milk CLA than late-growth or second-cutting forage (Chouinard et al., 1998b). Differences in digestibility between diets may explain some of this difference.

Feeding processed oilseeds or their oils have been shown to be particularly effective at increasing milk CLA concentrations. Kelly et al. (1998a) supplemented the basal diet with 53g/kg dry matter (DM) of peanut oil (high oleic acid), sunflower oil (high linoleic acid), or linseed oil (high linolenic acid). CLA concentrations were 13.3, 24.4, and 16.7 mg/g milk fat, respectively. The increase in CLA levels observed with the sunflower oil treatment represented levels approximately 500% greater than those typically seen in traditional diets. Chouinard et al. (1998a) fed diets supplemented with 4% DM of calcium salts of fatty acids from canola oil, soybean oil, or linseed oil. The resulting milk CLA concentrations were 13.0, 22.0, 19.0 mg/g fat for canola oil, soybean oil, and linseed oil respectively, and 3.5mg/g fat for control. Soybean oil, which is high in linoleic acid, was most effective at increasing the CLA. The linoleic acid most likely increases CLA by providing a substrate for CLA and trans 18:1 production in the rumen. It may also interfere with the biohydrogenation process. In vitro studies have shown that high levels of linoleic acid inhibit the conversion of trans-11 18:1 to 18:0 (Polan et al, 1964; Harfoot et al, 1973a; Noble et al., 1974). This would result in more trans-11 18:1 for tissue synthesis of CLA. It appears that the availability of the oils to the rumen microbes is also an important determinant of subsequent CLA production. Chouinard, et al (2001) showed that processing soybeans, especially by extrusion, increased milk CLA above that obtained by feeding ground soybeans. The extrusion process ruptures the seed, likely making the oil more available for rumen biohydrogenation. Feeding the raw unprocessed seed was not effective at increasing milk CLA (Chouinard et al 1998a; Dhiman et al, 2000). The amount and type of CLA isomers produced as a result of feeding supplemental fat varies to a large extent depending on the ruminal conditions. A study at Cornell University using supplemental fat found that the CLA levels in milk were halved when the forage:concentrate ratio of the diet was changed from 50:50 to 20:80 (Kelly and Bauman, 1996). Furthermore, as stated already, Griinari, et al. (1998) showed that high concentrate diets could alter the products of rumen biohydrogenation of polyunsaturated fatty acids resulting in an increase in the proportion of trans-10 18:1 and trans-10, cis-12 CLA isomers.

Dietary fish oil supplementation has also been found to increase the concentration of CLA in bovine milk from 0.2-0.6% of fatty acids in control diets to 1.5-2.7% of fatty acids in supplemented diets (reviewed in Chilliard, et al., 2001). This was somewhat surprising as fish oils are generally high in fatty acids of 20 or more carbons (especially 20:5 and 22:6) but low in 18 carbon polyunsaturated fatty acids. It is thought that the supplemental fish oils interfere with the biohydrogenation of 18:2 and 18:3 from the basal diet, specifically inhibiting the conversion of trans-11 18:1 to 18:0. Fish oil supplementation has been shown to increase ruminal production of trans-octadecanoic acids (Pennington and Davis, 1975; Wonsil, et al., 1994). Moreover, studies using fish supplementation that reported milk CLA values showed that the increase in CLA was almost exclusively in the cis-9, trans-11 isomer (Chilliard, et al., 1999; Offer et al., 1999). Feeding fish oil in combination with a source of 18:2 or 18:3 would therefore be expected to increase the level of milk CLA much more than would be achieved with 18:2 or 18:3 alone. This hypothesis was tested by Abu-Ghazaleh et al. (2002) who fed diets containing 0.5% fish oil, 2.5% soybean oil, or a combination of 0.5% fish oil and 2% soybean oil. They reported levels of cis-9, trans-11 CLA (g/100g fatty acids) of 0.33, 0.47, 0.79, and 1.39 for control, fish oil, soybean oil, and the combination, respectively. A similar result was obtained feeding 1% fish oil in combination with 2% high-linoleic acid processed sunflower seeds (Abu-Ghazaleh et al., 2003).

The ability of ionophores to affect the concentration of CLA in bovine milk fat has been evaluated. As stated previously ionophores appear to interfere with the rumen biohydrogenation of polyunsaturated fatty acids, probably by inhibiting the growth of gram positive bacteria. Fellner et al (1997) studied the effects of ionophores on lipid biohydrogenation using a continuous culture system. They found that the antiporter ionophores Monensin, Nigericin, and Tetronasin interfered with the biohydrogenation of linoleic acid. The study showed a reduction in the extent of linoleic acid biohydrogenation with an accumulation of the intermediate products of that process, including CLA. They followed up this work in dairy cows by evaluating the ability of monensin at 24 mg/kg of dietary dry matter to increase milk CLA over a 28-day period (Sauer et al. 1998). They observed a small but significant increase in CLA from 0.8% to 1.3% of milk fat. Other studies have failed to show a benefit of ionophores for enriching the concentration of CLA in milk fat (Dhiman, et al. 1999). The usefulness of ionophores to increase CLA in bovine milk is therefore considered equivocal. Furthermore, whether ionophores are effective over longer periods of time is also uncertain as the rumen has been known to adapt to the effects of ionophores (Griinari and Bauman, 1999).

1.3.5.2 Organoleptic characteristics of CLA-enriched dairy products

Changing the fatty acid composition of milk has the potential to change the organoleptic characteristics of the milk or milk product. For instance, milk with elevated levels of polyunsaturated fatty acids can be more susceptible to oxidation. Goering et al. (1976) showed that feeding protected safflower oil increased the 18:2 n-6 in milk fat and resulted in the development of strong oxidized flavors. The effect of increasing the concentration of CLA in milk on the organoleptic characteristics has not been thoroughly evaluated.

In studies from the Minesota-South Dakota Dairy Foods Research Center (Baer et al., 2001; Ramaswamy et al., 2001) four experienced panelists evaluated the flavor of

CLA-enriched milk (2.17 to 2.43 g CLA/100g fat) produced through fish oil and/or soybean feeding, compared to control milk (0.56 to 0.66 g CLA/100g fat). These studies concluded that there was no difference in flavor between the CLA-enriched milk and control. However, they appeared to be looking only for signs of oxidation. In these same studies, butter made from the CLA-enriched milk was softer and had flavor characteristics not different from control (Baer, et al., 2001; Ramaswamy, et al., 2001). In contrast to studies that evaluated milk enriched naturally in CLA, Campbell et al. (2003) carried out trained sensory evaluation on milk fortified with a synthetic mixture of CLA isomers. Nine panelists evaluated CLA-fortified milk and scored the milk based on four sensory characteristics, which they named "milkfat", "grassy/vegetable oil", "sweet taste", and "astringent" (Campbell et al., 2003). They found that the CLA-fortified milk had a significantly higher "grassy/vegetable oil" flavor and lower "milkfat" flavor compared to the 2% milk control. Since the researchers found no indicators of oxidation they attributed the particular flavor of CLA-fortified milk to the added CLA oil. In a consumer evaluation, the milk fortified with 2% CLA also scored lower for overall acceptability, flavor, and perceived freshness compared to the 2% milk fat control (Campbell et al., 2003). These results suggest that fortifying milk with synthetic CLA results in a product with less acceptable organoleptic characteristics.

1.3.5.3 Increasing Milk CLA Using Synthetic CLA

Conjugated linoleic acid can be synthesized from vegetable oils like sunflower using an alkaline-catalyzed procedure. As noted earlier, CLA produced in this way tends to contain a mixture of CLA isomers. This type of product has been shown to reduce subcutaneous fat and increase lean in pigs (Dugan et al., 1997) and will likely become commercially available in the near future. The trans-10, cis-12 isomer has been associated with these effects on body composition whereas the anticarcinogenic properties have been attributed to the cis-9, trans-11 and trans-10, cis-12 isomers (Pariza et al., 2001). Synthetic CLA could be used to increase the CLA concentration in bovine milk if protected in some way from the rumen environment. Methods available to reduce biohydrogenation in the rumen include encapsulation of the fat in formaldehyde-treated casein or feeding the fat as a calcium salt (Ashes et al., 1997).

Mixtures of CLA isomers have been found to have an inhibitory effect on milk fat synthesis (Loor and Herbein, 1999; Chouinard, et al., 1999, Mackle et al., 2003). The trans-10, cis-12 CLA appears to be the isomer responsible for this effect (Baumgard, et al., 2000). Abomasal infusion of trans-10, cis-12 at levels up to 14 g/day for five days produced a dose response reduction in milk fat yield and concentration in dairy cows (Baumgard, et al., 2001). Griinari et al (1999) have also shown that rumen concentrations of trans-10, cis-12 are negatively correlated with milk fat percentage in diets which cause milk fat depression. Decreases in acetyl Co-A carboxylase (ACC) and fatty acid synthase (FAS) activity and ACC mRNA abundance are associated with this depression in milk fat (Piperova, et al., 2000). The use of rumen-protected CLA isomers as a method of depressing milk fat may be useful as a tool to increase the protein to fat ratio in milk (Bauman and Griinari, 2001), and potentially improve the energy balance of early lactation cows (Perfield II et al., 2002). Feeding trials using calcium salts of CLA have demonstrated that they are an effective method of reducing milk fat percentage (Giesy et al., 1999; Sippel, et al., 2001). A study using goats showed that CLA could also be protected from rumen digestion by encapsulating the CLA in formaldehyde-treated casein (Gulati, et al., 2000). In view of the ability of the trans-10, cis-12 isomer to reduce body fat in animals (Dugan et al., 1997; Park, et al., 1997; DeLany et al., 1999; Ostrowska et al., 1999), interest has been shown in whether this isomer could have a benefit for weight reduction in humans. Feeding rumen-protected CLA could be a means of elevating the concentration of these fatty acids in bovine milk fat, thereby increasing the supply of these specific fatty acids in the human diet.

1.4 Conclusion

It has long been recognized that nutrition can have dramatic effects on both the content and composition of bovine milk fat. Dietary manipulation has been used to achieve various objectives in this regard including, causing a deliberate depression in milk fat for economic reasons, altering fat composition to improve processing or end product characteristics, or changing the concentration of saturated and unsaturated fatty acids for health reasons. Although the presence of CLA in milk fat has been known for several decades, the potential health benefits of CLA have been a more recent and unexpected discovery. Research in recent years has demonstrated the possibility of manipulating the concentration of CLA in milk fat through modification of the animal's diet. Producing CLA-enriched dairy products would allow people to increase their intake of CLA, without the need for drastic changes in food consumption patterns. It could also benefit the dairy industry by creating new market opportunities and enhancing the image of milk fat.

1.5 General Objectives

The general objective of this research was to generate new knowledge on the potential of dairy nutrition to enhance the concentration of CLA in bovine milk fat. In this regard, several experiments were carried out with the following goals:

1. To evaluate the potential to enhance CLA in milk fat using various feeding strategies aimed at increasing the natural synthesis of CLA in the cow.
2. To evaluate the organoleptic characteristics of CLA-enriched milk.
3. To evaluate the effectiveness of post-ruminal delivery of synthetically produced CLA as a means of elevating the milk fat concentration of CLA.

Table 1.1 Fatty Acid Composition of Various Oilseeds

Fatty acid[1]	Canola	Cottonseed	Linseed	Safflower	Soybean	Sunflower
16:0	4	25	5	7	8	6
18:0	2	3	3	2	3	4
18:1 n-9	52	17	20	9	24	20
18:2 n-6	25	54	16	80	58	66
18:3 n-3	13	-	55	<1	8	<1

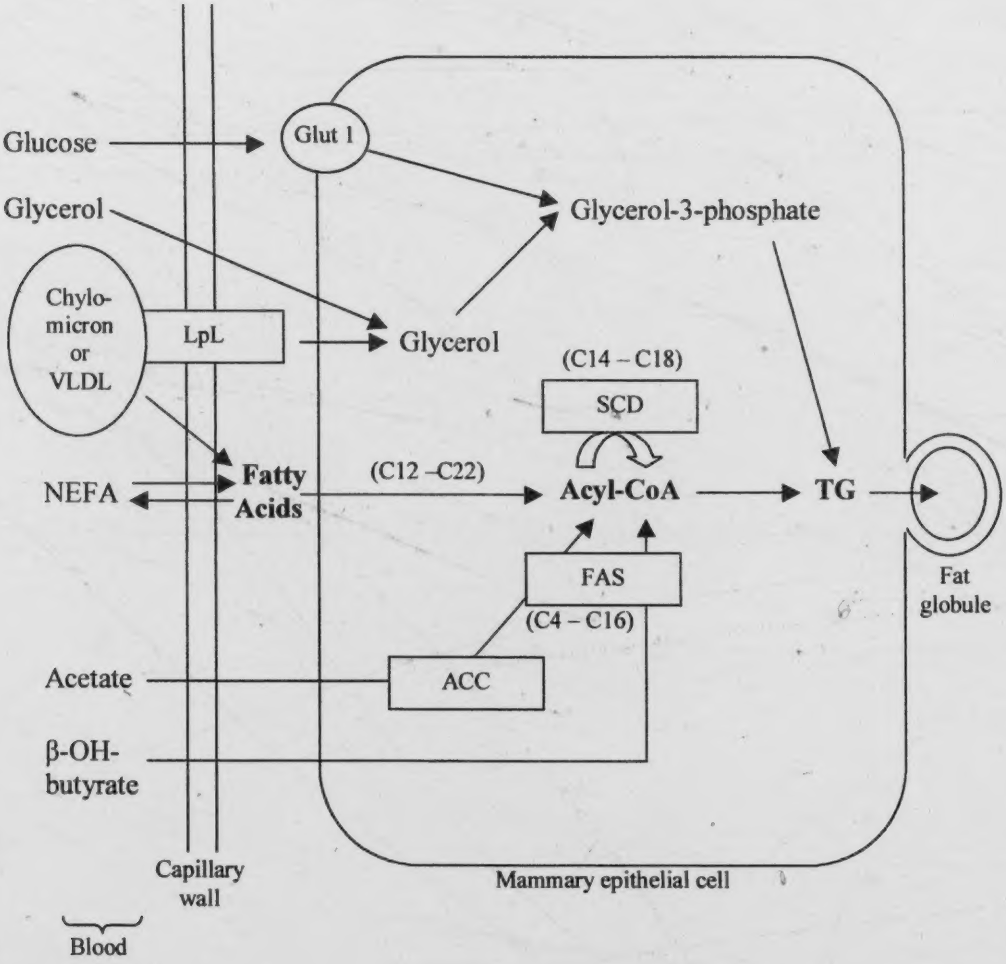
[1] Fatty acids expressed as a percentage of fatty acids
Source: Kennelly (1996)

Table 1.2 CLA content of various foods

Food	Total CLA concentration (mg/g fat)
Dairy products	
Homogenized	5.5
Butter fat	4.7
Mozzarella	4.9
Plain yogurt	4.8
Ice cream	3.6
Meats	
Ground beef	4.3
Lamb	5.6
Pork	0.6
Chicken	0.9
Salmon	0.3
Ground turkey	2.5

Source: Chin et al. (1992)

Figure 1.1 Milk fat synthesis and secretion in the ruminant mammary gland


Glucose
Glut 1
Glycerol
Glycerol-3-phosphate
Chylo-micron or VLDL
LpL
Glycerol
(C14 – C18)
SCD
NEFA
Fatty Acids
(C12 –C22)
Acyl-CoA
TG
FAS
(C4 – C16)
Fat globule
Acetate
ACC
β-OH-butyrate
Capillary wall
Mammary epithelial cell
Blood

Adapted from Chilliard et al. (2001a)

Figure 1.2 Formation of Conjugated Linoleic Acid in the Cow

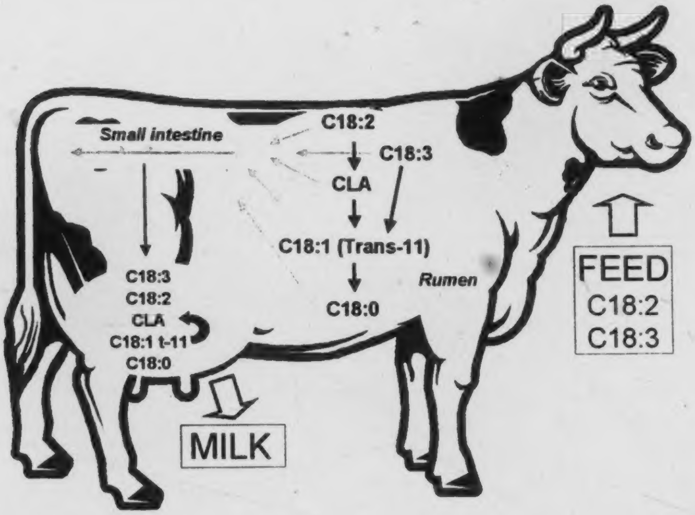
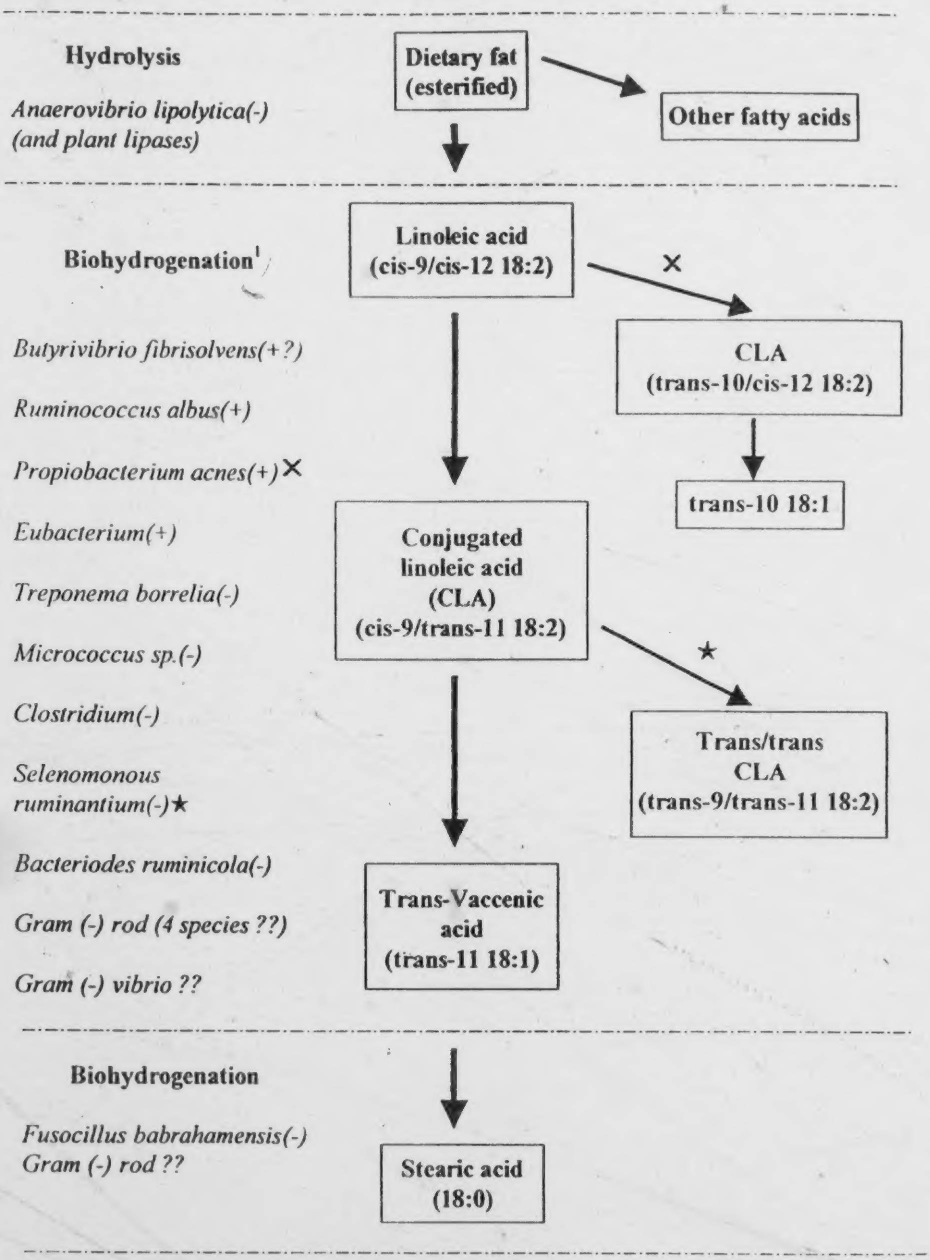

Small intestine
C18:2
C18:3
CLA
C18:1 (Trans-11)
C18:0
Rumen
C18:3
C18:2
CLA
C18:1 t-11
C18:0
MILK
FEED
C18:2
C18:3

Figure 1.3 Rumen biohydrogenation of linoleic acid


Hydrolysis
Anaerovibrio lipolytica(-)
(and plant lipases)
Dietary fat (esterified)
Other fatty acids
Biohydrogenation¹
Butyrivibrio fibrisolvens(+?)
Ruminococcus albus(+)
Propiobacterium acnes(+)×
Eubacterium(+)
Treponema borrelia(-)
Micrococcus sp.(-)
Clostridium(-)
Selenomonous ruminantium(-)★
Bacteriodes ruminicola(-)
Gram (-) rod (4 species ??)
Gram (-) vibrio ??
Linoleic acid (cis-9/cis-12 18:2)
×
CLA (trans-10/cis-12 18:2)
trans-10 18:1
Conjugated linoleic acid (CLA) (cis-9/trans-11 18:2)
★
Trans/trans CLA (trans-9/trans-11 18:2)
Trans-Vaccenic acid (trans-11 18:1)
Biohydrogenation
Fusocillus babrahamensis(-)
Gram (-) rod ??
Stearic acid (18:0)

Note: -/+ sign in parentheses indicate gram - or +
Note: ¹Bacteria carry out 18:2 n-6 to trans-11 18:1 conversion unless otherwise indicated, that is, ×★ indicate the specified bacterium is involved in a specific reaction as indicated

Adapted from:
Harfoot and Hazelwood (1988)

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Chapter 2 - Enhancing the concentration of conjugated linoleic acid in bovine milk fat: Manipulation of the cow's diet – feeding study I

2.1 Introduction

Conjugated linoleic acid (CLA) is the term given to a mixture of conjugated octadecadienoic acids of predominantly ruminant origin. The conjugated double bond configuration can be found on a range of positions on the carbon chain, with each double bond either in the *cis* or *trans* orientation. The predominant isomer in bovine milk fat is the cis-9, trans-11 18:2, which accounts for over 82% of the total (Chin et al., 1992). Although the presence of CLA in milk has been known for a long time (Kepler et al, 1966), current interest in this fatty acid has come from the more recent discovery of its health promoting properties (Pariza et al. 1985). Included among the wide array of CLA effects tested has been anticarcinogenic activity (Ha et al., 1990; Shultz et al, 1992; Ip et al 1994; Ip et al, 1999; Belury, 2002), antiatherogenic activity (Lee et al., 1994; Nicolosi et al., 1997), antidiabetic activity (Houseknecht et al. 1998; Belury, 2002), and antiobesity activity (Blankson et al., 2000; Smedman et al., 2001; Thom et al., 2001). Although the effects of CLA have mostly been demonstrated in animal models, it has been postulated that CLA could have similar beneficial effects in humans. Since CLA is primarily a product of ruminant animals, meat and milk from ruminants provides the best natural source of CLA in the human diet (Chin et al., 1992).

The CLA found in ruminant meat and milk appears to originate either directly from the rumen or by tissue desaturation of rumen-derived trans-11 18:1. In the rumen, isomers of CLA and trans-18:1 are produced as intermediates in the biohydrogenation of unsaturated fatty acids (Griinari et al., 1997). It has been suggested that little CLA actually accumulates in the rumen and that the majority of milk CLA is derived from trans-11 18:1 in the mammary gland through the action of Δ-9 desaturase (Corl, et al., 2000). Factors that increase the level of CLA or trans-11 18:1 synthesized in the rumen would therefore be expected to increase milk CLA.

Diet is by far the most influential factor determining the concentration of CLA in bovine milk fat. Feeding processed oilseeds or their oils have been shown to be particularly effective at increasing milk CLA concentrations (Kelly et al., 1998; Chouinard, et al., 2001). The reason for this lies in the high content of 18:2 n-6 (linoleic acid) and 18:3 n-3 (α-linolenic acid) of most oilseeds. Linoleic acid is hydrogenated in the rumen to CLA and trans-11 18:1. Alpha-linolenic acid goes through a similar biohydrogenation process producing trans-11 18:1, but does not appear to produce CLA as an intermediate. The conversion of trans-11 18:1 to 18:0 appears to occur at a lower rate resulting in an accumulation of ruminal trans-11 18:1 (Griinari et al., 1997).

Factors that interfere with the biohydrogenation pathway leading to a greater accumulation of the intermediate products might also be expected to increase milk CLA. Fellner et al (1997) studied the effects of ionophores on lipid biohydrogenation using a continuous culture system. They found that the antiporter ionophores Monensin, Nigericin, and Tetronasin interfered with the biohydrogenation of linoleic acid. The study showed that ionophores caused a reduction in the extent of linoleic acid biohydrogenation with an accumulation of intermediate products, including CLA. They followed up this work in dairy cows by evaluating the effect of monensin at 24 mg/kg of dietary dry matter on milk CLA over a 28-day period (Sauer et al. 1998). They observed a small but significant increase in CLA from 0.8% to 1.3% of milk fat. Other studies have failed to show a benefit of ionophores for enriching the concentration of CLA in milk fat (Dhiman, et al. 1999). The usefulness of ionophores to increase CLA in bovine milk is therefore considered equivocal.

The objective of this study was to evaluate the potential of dietary modification to increase the level of CLA in bovine milk fat. It was hypothesized that feeding safflower oil would significantly increase milk CLA due to its very high content (76%) of 18:2 n-6. It was also hypothesized that adding monensin in combination with safflower oil would increase milk CLA more than safflower alone through the action of monensin on the biohydrogenation process, as demonstrated *in vitro* (Fellner et al., 1997).

2.2 Materials and Methods

2.2.1 Animals and treatments

All procedures involving the use of animals were approved by the Faculty Animal Policy and Welfare Committee at the University of Alberta. Twenty-eight lactating Holstein cows (eight primiparous, twenty multiparous) were used in a randomized complete block design with repeated measures. The animals averaged 213 ± 61 DIM at the start of the trial with an average body weight (kg) of 607 ± 57.8 and an average body condition score (5-point scale) of 2.77 ± 0.46. All cows were first fed a control (CTL) diet for eight days (week 0). Animals were then blocked according to parity and DIM and randomly placed into one of four groups. Each group was fed one of four diets for a 15 day treatment period: (1) Control diet (CTL); (2) Control diet including monesin supplemented at 24 ppm of DM (MON); (3) Control diet including safflower oil supplemented at 6% of DM (SAFF); (4) Control including safflower oil supplemented at

6% of DM plus monesin supplemented at 24 ppm of DM (SAFF/M). All diets were formulated to meet or exceed NRC recommendations (NRC, 1989).

Cows were housed in tie-stalls with water available at all times. The diets were fed once per day at nine AM as a TMR consisting of 60% forage and 40% concentrate (Table 2.1). Feed intake was recorded daily and adjusted to maintain 5 to 10% orts. Milking was carried out twice per day starting at 0330 and 1430. Milk yield was recorded daily.

2.2.2 Sampling and Analysis of Feed and Milk

Samples of TMR, orts and ingredients were taken twice weekly. Each sample was immediately dried at 60 °C for 72 h. Prior to analysis samples were ground to pass through a 1 mm screen using a Model 4 Thomas-Wiley Laboratory Mill (Arthur H. Thomas Co., Philadelphia, PA, USA). True dry matter (DM) was determined by drying samples to a constant weight at 110 °C (True DM = (DM % after 60°C) x (DM % after 110 °C)/100). Following drying at 110 °C, ash content was determined by ashing at 500 °C for at least 8h. Samples were also analyzed for crude protein (CP) (Leco® Corporation, St. Joseph MI, USA), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Van Soest et al., 1991), and crude fat (Elliot et al, 1999).

Milk was sampled from each cow at AM and PM milking on the last two days of week 0 and the last two days of the treatment period. The amount sampled at each milking was proportional to the milk yield. The AM and PM samples were then combined to give one sample for each cow on each sampling day. A portion of milk from each cow was preserved with potassium dichromate and analyzed for protein, fat, lactose, and somatic cell count at the Alberta Agriculture, Food and Rural Development Central Milk Testing Laboratory (Edmonton, Alberta, Canada). The rest of the milk was stored at –20 °C for later analysis.

Lipids for fatty acid analysis were extracted from the milk using chloroform:methanol (2:1) (Folch et al, 1957). The milk was thawed at 27 °C. A 2ml sample of the thawed milk was placed in a 50ml tube. Twenty-four milliliters of chloroform:methanol (2:1) were added. The tube was capped, shaken for 30 seconds by hand and allowed to stand for at least one hour. Eight milliliters of 0.8% NaCl w/v were added and the tube let stand overnight at 4 °C. The tube was then warmed to room temperature and centrifuged at approximately 2500rpm for 5min. The upper methanol:water layer was removed using a water aspirator and discarded. Ten milliliters of the lower chloroform phase were transferred to a 20ml-glass scintillation vial. The choroform was evaporated at 40 ^{0}C under nitrogen leaving the fat (~20 to 30 mg).

The fatty acids were then methylated by sodium methoxide using a procedure similar to that described by Chouinard et al (1999), which was based on the method of Christie (1982). Two milliliters of hexane were added to the 20ml glass scintillation vial to resolubilize the fat. From this, a volume representing approximately 5mg of fat was removed and placed in a 14ml screw top test tube. Twenty micro-liters of methyl acetate were added. Following vortexing 40µl of methylating agent (0.5M sodium methoxide) was added. The mixture was vortexed and allowed to react for 10 min at room temperature. The reaction was stopped with 30µl of termination reagent (1g oxalic acid in 30ml diethyl ether). The sample was then centrifuged at 2400xg (3200rpm) for 5 minutes leaving a clear layer of hexane from which an aliquot was taken for GC analysis.

The fatty acid methyl esters were analyzed on a Varian 3600 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) with a temperature programmable injector and flame ionization detector. Separation of the fatty acid methyl esters was performed using an CP-Sil 88 fused silica capillary column (50m x 0.25mm (i.d.), with 0.25μm film thickness) (Chrompack, Middelburg, The Netherlands). Purified Helium (Praxair, Edmonton, Canada) was used as the carrier gas with a head pressure of 25 psi and a flow rate of 1ml/min. The initial column temperature was set at 50°C and held for 0.1 min, increased to 180°C at 25°C per min and held for 1 min, further increased to 190°C at 2°C per min and held for 2 min, and finally increased to 230°C at 10°C per min and held for 7 min. The initial injector temperature was 90°C, increasing at 150°C per min to 240°C and held for 23 min. The detector temperature was set at 240°C. Peak area was measured using the Scimadzu Class-VP chromatography data system (Shimadzu Scientific Instruments Inc, Columbia, MD, USA). Peaks were identified using Nu Chek Prep standards #85 and #411 (Nu Chek Prep, Elysian, MN, USA). Conjugated linoleic acids isomers were identified using standards from Matreya (Matreya, Inc., PA, USA). Detector response for individual fatty acids was verified using Nu Chek Prep standard #60 (Nu Chek Prep, Elysian, MN, USA). Each fatty acid was reported as a percentage of fatty acid methyl esters.

2.2.3 Statistical Analysis

Data was analyzed statistically as a randomized block design with a repeated measures treatment structure using the MIXED procedure of SAS version 8.3 (SAS Institute, Cary, NC). Cow within treatment was the experimental unit with week of sampling as the repeated measure. Treatment, week, block, and treatment by week interaction were fixed effects and cow was a random effect. The Kenward-Roger option was used to estimate denominator degrees of freedom. The variance-covariance matrix structure was chosen for each statistical model in a process wherein the best fit was chosen based on the Schwarz's Bayesian Criterion. Least square means were estimated and separated using the *pdiff* option when fixed effects were significant ($P<0.05$).

2.3 Results and Discussion

2.3.1 Dry Matter Intake, Milk Yield, and Milk Composition

The effect of diet on dry matter intake (DMI), milk yield, and milk composition is presented in Table 2.2. Dry matter intake and milk yield were not significantly different between treatments. However, both percentage and yield of fat were significantly affected by treatment. The SAFF and SAFF/M treatments produced milk with a lower milk fat percent than CTL. A similar though non-significant ($P=0.054$) effect was also observed for MON compared to CTL. A decrease in both yield and concentration of milk fat with dietary inclusion of polyunsaturated fat is a common finding and is widely accepted as being caused by rumen-derived trans-10 fatty acids (Bauman and Griinari, 2001). The milk fatty acid profile in this study supports the trans-10 hypothesis in that the milk from SAFF and SAFF/M had significantly higher levels of trans-10, cis-12 18:2, compared to CTL and MON (Table 2.3). A significantly higher level of trans-10, cis-12 was also observed in SAFF/M compared to SAFF. This is in agreement with Jenkins et al. (2003) who reported that monensin increased the level of trans-10 18:1 compared to a control in *in vitro* continuous cultures fed barley grain. This effect of monensin might explain the trend towards a decrease in milk fat with MON compared to CTL (Table 2.2). Milk fat depression is often characterized by a reduction in the mammary synthesis of short to medium chain fatty acids (4:0 to 16:0) (Drackley et al, 1992; Delbecchi et al., 2001), which was also observed in this study (Table 2.3).

Although diets supplemented with oils often decrease milk protein percent (Drackley and Elliot, 1993; Elliot et al, 1993; Wu et al, 1994), protein percent and yield were not significantly different between treatments in this study. Concentration and yield of lactose was also not different among treatments.

2.3.2 Milk Fatty Acid Composition

The CTL diet, representative of diets fed in Alberta, resulted in milk fat with a cis-9, trans-11 CLA concentration of 0.45%, similar to that typically reported for whole milk (Table 2.3). Cows fed the SAFF diet produced milk fat with 3.36% cis-9, trans-11 CLA, significantly greater than CTL. Although monensin (MON) alone had no significant effect on milk cis-9, trans-11, monensin in combination with safflower oil increased milk fat CLA more than safflower alone. The concentration of cis-9, trans-11 CLA with SAFF/M was 5.15%, more than ten times the level in CTL milk. Although the yield of fat was lower in the SAFF/M treatment, the yield of CLA was still approximately nine times greater than the yield of CLA with CTL (Table 2.3). The SAFF and SAFF/M diets also increased the levels of trans-10, cis-12 and trans/trans isomers of CLA compared to CTL and MON. The cis-9, trans-11 isomer represented over 90% of the CLA in milk, which is comparable to values reported previously (Chin et al., 1992). This study agrees with other published data that showed that oils high in 18:2 n-6 were effective at increasing milk CLA (Chouinard et al., 1998; Kelly et al., 1998). Kelly et al. (1998) found that a diet supplemented with 53g/kg dry matter of sunflower oil produced milk fat with CLA concentrations of 24.4 mg/g fat (approximately equivalent to 2.44%). Although this is lower than the result found the present study, this might be explained by a number of factors. The present study fed a higher level (6% verses 5.3% DM) of an oil that had a higher level of 18:2 n-6 (76% verses 71%). In addition to this, the present study also used monensin, which had an additive effect compared to safflower alone. In fact, this study clearly demonstrated the effectiveness of safflower oil to increase milk concentrations of CLA. Furthermore, it provided support for *in vitro* data (Fellner et al., 1997) indicating that monensin could inhibit rumen biohydrogenation with a resultant greater concentration of intermediates like CLA.

An important question is whether the degree of enrichment achieved is sufficient to provide adequate intake of CLA at moderate levels of milk consumption. Intake of CLA in North America has been estimated at 52 to 137mg CLA/day (Ritzenenthaler et al 1998). Ip, et al. (1994) suggested that the level of CLA intake necessary to produce anti-carcinogenic effects in humans might be about 3g per day based on direct extrapolation from animal studies. Almost a decade later, this figure of 3g is still being reported as the target intake (Campbell et al., 2003), although others have suggested that direct extrapolation may be an overestimation (Ma, et al., 2000). Extrapolating on an energy basis rather than a weight basis, Ma, et al. (2000) suggested that 600 mg may be a more realistic estimate of a beneficial intake for humans. Using the CLA percentage achieved with the SAFF/M diet (Table 2.3), one serving of whole milk (420mg CLA) and a sandwich with butter (334mg CLA) and cheddar cheese (660mg CLA) would provide

1414 mg (1.414g) CLA. This example illustrates how CLA enriched milk and milk products could supply dietary CLA at levels that may benefit health, without the need for unrealistic changes to eating habits.

Cows fed the SAFF and SAFF/M diets had significantly higher levels of trans-18:1 in their milk compared to CTL and MON (Table 2.3). Safflower in combination with monensin also had a significantly greater effect on milk trans-18:1 than safflower alone. The higher trans-18:1 observed with SAFF/M compared to SAFF might suggest that the monensin was interfering with the rumen biohydrogenation by preventing the conversion of trans-11 18:1 to 18:0. This would explain the higher CLA observed with SAFF/M as higher trans-11 18:1 would provide more substrate for mammary CLA synthesis through Δ-9 desaturation. If the theory that the conversion of trans 18:1 to 18:0 was inhibited by monensin is correct, then we might expect to see a lower level of 18:0 in SAFF/M milk compared to SAFF. In fact, a strong trend (*P*=0.07) was observed towards lower 18:0 in SAFF/M compared to SAFF milk.

In the past decade there has been an accumulation of evidence that suggests that trans fatty acids (TFA) may contribute to the development of coronary heart disease (CHD). Investigations found that TFA increased blood cholesterol levels, which are believed to be an important risk factor for CHD. This was supported by strong epidemiological evidence. A study reported by Willett et al. (1993), which followed more than 80,000 women for 8 years, found an association between high intakes of TFA and coronary heart disease. This created the impetus for plans to make labeling of TFA on food packaging mandatory. However, the study reported by Willett et al (1993) showed that the association between TFA and CHD was specific for TFA from industrial hydrogenated fats, whereas TFA of animal origin were not correlated with CHD.

Estimates of TFA intake in the US range from as low as 2.6 g/d to as much as 12.8 g/d (Kris-Etherton et al., 1995). A seven-country study estimated intake of TFA at between 0.1 g/d and 5.5 g/d (Kromhout et al., 1995). Approximately 75 to 80% of the TFA in our diet comes from partially hydrogenated vegetable oils like those found in baked foods, certain types of margarine, and foods that are deep fat fried (Allison et al., 1999). The composition of trans isomers from these sources is different from trans fatty acids of ruminant origin, which may provide a rationale for the differences seen in the epidemiological studies (Kris-Etherton et al., 1995; Watts et al., 1996; Matthan et al., 1999). The primary trans fatty acids in bovine milk are 18:1 trans-11 and CLA, whereas partially hydrogenated vegetable oils are characterized by a range of trans fatty acids such as 18:1 trans-8, trans-9, trans-10, trans-11, trans-12 and trans-13.

A study by Hudgins et al. (1991) correlated concentrations of individual fatty acids in human adipose tissue with various risk factors for cardiovascular disease. They found no evidence for strong associations between cardiovascular risk factors and adipose tissue concentrations of cis and trans fatty acids. In fact, a significant negative correlation was reported between adipose trans-11 18:1 and blood low-density-lipoprotein (LDL) cholesterol concentration (Hudgins et al., 1991). As noted earlier, CLA has been found to inhibit cholesterol-induced atherosclerosis in rabbits and hamsters (Lee et al., 1994; Nicolosi et al., 1997). Furthermore, there is evidence that trans-11 18:1 can be desaturated to cis-9, trans-11 CLA in human tissues (Salminen et al. 1998). Clearly, the issue of TFA in food is controversial and requires further investigation. The planned changes in food labeling to include TFA, along with the current nutritional advice suggesting a decrease in TFA intake is sure to present a confusing message to consumers with the promotion of "high-trans" CLA-enriched milk. This is an important issue that needs to be considered with the development of CLA-enriched dairy products.

Even though dairy products contribute only about 15 to 25% of the total fat in the Western diet, they provide 25 to 35% of the total saturated fat (Chilliard et al., 2000). Ruminant fat has been associated with an elevation in blood cholesterol because of its high content of saturated fatty acids, and particularly because of its high content of 14:0 and 16:0, which are generally considered hyper-cholesterolemic (Berner, 1993). In this study we found that the diets that increased CLA also resulted in a decrease in the proportion of 14:0 and 16:0 in milk fat. The SAFF and SAFF/M milk compared to CTL or MON had approximately 33 to 35% lower 14:0, and 41 to 44% lower 16:0. There was also an increase of approximately 50% in 18:1 *cis*-9 levels. This could also be considered a positive change as 18:1 *cis*-9 in the diet is considered to have a cholesterol lowering effect (Berner, 1993). The level of 18:2 n-6 was significantly increased with SAFF and SAFF/M although the level of this fatty acid was still relatively low in the milk fat. This is undoubtedly because of the extensive biohydrogenation that occurs in the rumen (Doreau and Ferlay, 1994).

2.4 Conclusion

This study demonstrated that CLA could be substantially enhanced in bovine milk fat through modification of the dairy diet. Since 18:2 n-6 is a substrate for rumen synthesis of CLA and/or trans-11 18:1, feeding safflower oil (76% 18:2 n-6) significantly increased milk fat CLA. Furthermore, monensin had an additive effect so that the combination of safflower with monensin raised CLA levels more than that achieved by safflower alone. In addition to the increase in CLA, diets including safflower or safflower with monensin resulted in other favorable changes in the fatty acid profile such as a decrease in 14:0 and 16:0, and an increase in 18:1 n-9.

Table 2.1 Ingredient and chemical composition of experimental diets.

	Treatment[1]			
Item	CTL	MON	SAFF	SAFF/M
	(% of DM)			
Ingredient composition				
Barley silage	25	25	25	25
Alfalfa silage	25	25	25	25
Alfalfa hay	10	10	10	10
Ground corn	20.5	20	13	12.5
Barley	10.4	10.4	10.4	10.4
Soybean meal	7	7	8.5	8.5
Safflower oil[2]	0	0	6	6
Monensin[3]	0	0.54	0	0.54
Limestone	0.6	0.6	0.6	0.6
Mineral salt[4]	0.4	0.4	0.4	0.4
Dicalcium phosphate	0.40	0.40	0.40	0.40
Magnesium oxide	0.28	0.28	0.28	0.28
Salt	0.2	0.16	0.2	0.16
Sodium bicarbonate	0.15	0.15	0.15	0.15
Vitamin ADE[5]	0.07	0.07	0.07	0.07
Chemical composition				
DM, %	44.7	44.7	45.2	45.8
CP	17.3	17.3	17.0	16.9
Crude fat	4.34	4.51	7.74	7.58
NDF	44.1	45.1	46.7	48.0
ADF	24.8	26.6	27.9	27.9
Ash	10.3	10.4	9.9	10.1
NE_L[6], Mcal/Kg	1.60	1.59	1.83	1.82

[1] Diets: Control (CTL), and control supplemented with, monensin (Mon), safflower oil (SAFF), safflower oil plus monensin (SAFF/M).

[2] Contained: 18:2 n-6 (75.9%), 18:1 n-9 (12.5%), 18:0 (1.9%), and 16:0 (7.0%).

[3] Contained: monensin (4,400 mg/kg).

[4] Contained: salt (min 95%), iodine (150 mg/kg), cobalt (50 mg/kg), copper (3500 mg/kg), manganese (10,000 mg/kg), zinc (9,000 mg/kg), and selenium (75 mg/kg).

[5] Contained: vitamin A (min 10,000,000 IU/kg), vitamin D (min 1,000,000 IU/kg), vitamin E (min 10,000 IU/kg).

[6] Estimated from NRC (1989)

Table 2.2. Dry matter intake (DMI), milk yield, and milk composition from cows fed control diet (CTL), control including monensin (MON), control including safflower oil (SAFF), or control including safflower oil and monensin (SAFF/M).

	Treatments[1]				
	CTL	MON	SAFF	SAFF/M	sem
DMI, kg/d	20.45	20.25	18.39	19.52	0.74
Milk yield, kg/day[2]	26.87	27.58	26.78	27.83	1.48
Fat, %[3]	4.02^a	3.57^a	2.83^b	2.95^b	0.16
Fat yield, kg/day	1.05^a	0.97^a	0.74^b	0.81^b	0.05
Protein, %	3.32	3.38	3.11	3.23	0.10
Protein yield, kg/day	0.87	0.92	0.82	0.88	0.05
Lactose, %	4.30	4.54	4.26	4.50	0.09
Lactose yield,	1.13	1.24	1.14	1.24	0.08

^{a,b,c}Within a row, values with different superscripts are significantly different ($P < 0.05$).

[1] Diets were control (CTL), and control supplemented with, monensin (Mon), safflower oil (SAFF), safflower oil plus monensin (SAFF/M).

[2] Average milk yield over the last eight days of the treatment period.

[3] Values for fat, protein, and lactose represent average values for milk taken from the last two days of the treatment period.

Table 2.3. Fatty acid composition of milk fat from cows fed control diet (CTL), control including monensin (MON), control including safflower oil (SAFF), or control including safflower oil and monensin (SAFF/M).

	Treatments[1]				
Fatty acid[2]	CTL	MON	SAFF	SAFF/M	sem
4:0	5.17^{a}	4.82^{a}	5.08^{a}	4.06^{b}	0.20
6:0	3.38^{a}	3.20^{a}	2.48^{b}	2.14^{b}	0.12
8:0	1.98^{a}	1.87^{a}	1.24^{b}	1.13^{b}	0.06
10:0	4.23^{a}	4.20^{a}	2.18^{b}	2.06^{b}	0.15
11:0	0.65^{a}	0.61^{a}	0.26^{b}	0.23^{b}	0.03
12:0	4.80^{a}	4.79^{a}	2.44^{b}	2.40^{b}	0.13
13:0	0.25^{a}	0.31^{b}	0.14^{c}	0.15^{c}	0.02
14:0	13.78^{a}	14.17^{a}	9.09^{b}	9.16^{b}	0.23
14:1	1.36^{ad}	1.44^{ac}	0.84^{b}	1.01^{bd}	0.12
15:0	1.68^{a}	1.92^{b}	0.97^{c}	0.98^{c}	0.06
16:0	33.36^{a}	32.25^{a}	18.90^{b}	18.66^{b}	0.90
16:1 n-7	1.87^{a}	1.96^{a}	1.11^{b}	1.20^{b}	0.17
18:0	5.73^{a}	5.21^{a}	8.98^{b}	8.02^{b}	0.36
18:1 trans	1.40^{a}	1.54^{a}	9.56^{b}	13.53^{c}	0.76
18:1 n-12	0.71^{a}	0.96^{a}	1.88^{b}	1.50^{c}	0.09
18:1 n-9	11.59^{a}	12.05^{a}	18.47^{b}	16.72^{c}	0.47
18:1 n-7	0.61^{a}	0.63^{a}	0.86^{b}	0.83^{b}	0.02
18:2 t-11, c-15	0.16^{a}	0.18^{a}	0.42^{b}	0.40^{b}	0.01
18:2 n-6	1.38^{a}	1.49^{a}	2.69^{b}	2.58^{b}	0.08
18:3 n-3	0.39^{ab}	0.41^{a}	0.35^{bc}	0.34^{c}	0.02
20:0	0.12^{a}	0.11^{a}	0.15^{b}	0.14^{ab}	0.01
20:1 n-12	0.10	0.11	0.12	0.11	0.01
20:1 n-9	0.03^{a}	0.03^{a}	0.06^{b}	0.07^{c}	0.003
CLA c-9, t-11[3]	0.45^{a}	0.52^{a}	3.36^{b}	5.15^{c}	0.23
CLA t-10, c-12	nd^{a}	nd^{a}	0.05^{b}	0.08^{c}	0.01
CLA trans/trans	0.03^{a}	0.04^{a}	0.13^{b}	0.15^{b}	0.01
Other FA	4.78^{a}	5.18^{a}	8.18^{b}	7.17^{c}	0.16
CLA c-9, t-11 yield, g/d	4.70^{a}	4.95^{a}	25.79^{b}	41.97^{c}	3.23

[a,b,c] Within a row, values with different superscripts are significantly different ($P < 0.05$).

[1] Diets were control (CTL), and control supplemented with, monensin (Mon), safflower oil (SAFF), safflower oil plus monensin (SAFF/M).

[2] All values presented as percentage of fatty acid methyl esters.

[3] The chromatography methods used were unable to separate the minor quantities of 7/9 and 8/10 CLA isomers from the main cis-9, trans-11 isomer.

nd = Not detected

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Chapter 3 - Enhancing the concentration of conjugated linoleic acid in bovine milk fat: Manipulation of the cow's diet – feeding study II

3.1 Introduction

Conjugated linoleic acid (CLA) refers to a mixture of 18-carbon conjugated dienoic acids, of which the cis-9, trans-11 is the most common naturally occurring isomer. Mixtures of CLA can be synthesized from linoleic acid (18:2 n-6), and are found naturally in the meat and milk of ruminant animals (Chin et al., 1992). Certain bacteria within the rumen of cows, goats, and sheep are capable of converting 18:2 n-6 to CLA and trans 18:1. Rumen-escape of CLA, as well as tissue desaturation of trans-11 18:1 by stearoyl-CoA desaturase (SCD), result in the presence of these fatty acids in ruminant adipose and milk fat (Griinari and Bauman, 1999). Isomers of CLA have been associated with a range of biological properties including, anticarcinogenic activity (Ha et al., 1990; Shultz et al, 1992; Ip et al 1994; Ip et al, 1999; Belury, 2002), antiatherogenic activity (Lee et al., 1994; Nicolosi et al., 1997), antidiabetic activity (Houseknecht et al. 1998; Belury, 2002), and antiobesity activity (Blankson et al., 2000; Smedman et al., 2001; Thom et al., 2001). Interest in increasing CLA in bovine milk has come from the anticipation that the consumption of milk with elevated CLA will have benefits for human health.

Feeding processed oilseeds, or their oils, to dairy cows have been shown to increase milk CLA concentrations (Kelly et al., 1998; Chouinard, et al., 2001). More specifically, oilseeds that are characterized by a high concentration of 18:2 n-6, such as soybean and sunflower, have been particularly effective (Kelly et al., 1998). The reason for this lies in the fact that 18:2 n-6 undergoes extensive biohydrogenation in the rumen yielding CLA and trans-11 18:1 as intermediates. In a previous study (Chapter 2), we found that feeding safflower oil (76% 18:2 n-6) at 6% of DM increased milk fat CLA. Furthermore, feeding monensin at 24 ppm along with safflower oil appeared to have an additive effect so that the combination of safflower with monensin raised CLA levels more than safflower alone. This previous work demonstrated that milk fat CLA concentrations could be enhanced by as much as ten times through modification of the dairy diet.

The study presented here was carried out to follow up on the work presented in chapter two. The initial study involved feeding the cows over a two-week period. However, there are questions surrounding the long-term effectiveness of monensin due to the adaptation of rumen microbes to monensin (Griinari and Bauman, 1999; Chilliard et al., 2000). One objective of this study was to evaluate the effectiveness of monensin over a longer (two-month) period. A second objective was to evaluate the effect of supplemental vitamin E on milk CLA concentrations. Previous researchers have shown that dietary vitamin E was capable of reducing the extent of dietary-induced milk fat depression (Charmley and Nicholson, 1993; Charmley and Nicholson, 1994; Focant et al., 1998). If milk fat depression is caused by rumen synthesized trans-10 fatty acids as proposed by Bauman and Griinari (2001), it is possible that the alleviation of this depression by vitamin E is brought about by a shift in rumen biohydrogenation towards pathways that produce trans-11, and away from trans-10. Apart from a decrease in milk fat depression, this could also provide more trans-11 18:1 for mammary CLA synthesis. Vitamin E would also protect the milk from oxidation that could be a problem with higher concentrations of polyunsaturated fatty acids in the milk (see Chapter 4). Another specific aim of this experiment was to evaluate the effectiveness of flaxseed oil to increase milk CLA. It was hypothesized that flaxseed oil would significantly increase milk CLA because of its high content of 18:3 n-3, a precursor of rumen synthesized trans-11 18:1. This study was designed to directly compare the effectiveness of flaxseed oil against safflower oil for enhancing milk CLA. A final objective was to measure the mammary expression of stearoyl-CoA desaturase (SCD). It was hypothesized that variation within treatment groups could be partly explained by genetic variation such as a variation in mammary SCD expression. This study aimed to test this hypothesis by correlating mammary SCD mRNA with the ratio of cis-9, trans-11 to trans-11 18:1. It was hypothesized that cows (within a treatment group) with a higher expression of SCD would have a higher ratio of cis-9, trans-11 to trans-11 18:1.

3.2 Materials and Methods

3.2.1 Animals and Treatments

All procedures involving the use of animals were approved by the Faculty Animal Policy and Welfare Committee at the University of Alberta. Twenty-eight primiparous and 34 multiparous lactating Holstein cows were used in a randomized block design with repeated measures. Animals were blocked according to parity and DIM. Cows within each block were then randomly assigned to one of six treatment diets: (1) Control diet (CTL); (2) Control diet including safflower oil supplemented at 6% of DM (SAFF); (3) Control diet including safflower oil supplemented at 6% of DM plus Vitamin E supplemented at 150 IU/kg DM (SAFF/E); (4) Control diet including safflower oil supplemented at 6% of DM plus monensin supplemented at 24 ppm of DM (SAFF/M);

(5) Control diet including safflower oil supplemented at 6% of DM plus Vitamin E supplemented at 150 IU/kg DM plus monensin supplemented at 24 ppm of DM (SAFF/M/E); (6) Control diet including flaxseed oil supplemented at 6% of DM plus Vitamin E supplemented at 150 IU/kg DM (FLAX/E). The supplementary ingredients were added to their respective concentrates prior to addition of forages by thorough mixing in Calan data rangers in 500 kg batches. All diets were formulated to meet or exceed NRC recommendations (NRC, 1989).

Cows were housed in tie-stalls with water available at all times. The diets were fed once per day at nine AM as a TMR consisting of 60% forage and 40% concentrate (Table 3.1, 3.2). Feed intake was recorded daily and adjusted to maintain 5 to 10% orts. The CTL diet was fed initially to all cows for 10 days (week 0). Cows then received their respective diets for a period of nine weeks. Cows were adapted to dietary change over a three-day period. Milking was carried out twice per day starting at 0330 and 1430. Milk yield was recorded daily. Body weight was recorded once per week after AM milking and body condition score was estimated on a 5-point scale at the end of weeks 0, 3, 6, and 9.

3.2.2 Sampling and Analysis of Feed and Milk

Samples of TMR and feed ingredients were taken once on each of weeks 0, 2, 4, 6, and 8. Orts from each animal were sampled once on each of weeks 0, 4, and 8. Each TMR, ingredient and orts sample was immediately dried at 60 °C for 72 h. Prior to analysis samples were ground to pass through a 1 mm screen using a Model 4 Thomas-Wiley Laboratory Mill (Arthur H. Thomas Co., Philadelphia, PA, USA). True dry matter

(DM) was determined by drying samples to a constant weight at 110 °C {True DM = (DM % after 60°C) x (DM % after 110 °C)/100}. Following drying at 110 °C, ash content was determined by ashing at 500 °C for at least 8 h. Samples were also analyzed for crude protein (CP) (Leco® Corporation, St. Joseph MI, USA), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Van Soest et al., 1991), and crude fat (Elliot et al, 1999).

Milk was sampled from each cow at AM and PM milking on the last day of weeks 0, 2, 4, and 8. The amount sampled at each milking was proportional to the milk yield. The AM and PM samples were then combined to give one sample for each cow at each time point. A portion of milk from each cow was preserved with potassium dichromate and analyzed for protein, fat, lactose, and somatic cell count at the Alberta Agriculture, Food and Rural Development Central Milk Testing Laboratory (Edmonton, Alberta, Canada). The rest of the milk was stored at –20 °C for later analysis.

Lipids for fatty acid analysis were extracted from the milk using chloroform:methanol (2:1) (Folch et al, 1957). The milk was thawed at 27 °C. A 2ml sample of the thawed milk was placed in a 50 ml tube. Twenty-four milliliters of chloroform:methanol (2:1) were added. The tube was capped, shaken for 30 s by hand and allowed to stand for at least one hour. Eight ml of 0.8 % NaCl were added and the tube let stand overnight at 4 °C. The tube was warmed to room temperature and centrifuged at approximately 2500 rpm for 5 min. The upper methanol:water layer was then removed using a water aspirator and discarded. Ten milliliters of the lower chloroform phase were transferred to a 20 ml glass scintillation vial. The choroform was evaporated at 40 °C under nitrogen leaving the fat (~20 to 30 mg).

The fatty acids were then methylated by sodium methoxide using a procedure similar to that described by Chouinard et al (1999), which was based on the method of Christie (1982). Two milliliters of hexane were added to the 20 ml glass scintillation vial to resolubilize the fat. The hexane containing the fat was then transferred into a 14 ml screw-top tube. Forty µl of methyl acetate were added. Following vortexing 80 µl of methylating agent (0.5 M sodium methoxide) were added. The mixture was vortexed and incubated for 15 min in a 50 °C water bath. The reaction was stopped by adding 60 µl of termination reagent (1 g oxalic acid in 30 ml diethyl ether). Two ml of water were added to remove any non-lipid material. The sample was vortexed and centrifuged at 2400 xg (3200 rpm) for 5 min leaving a clear layer of hexane from which an aliquot was taken for GC analysis.

The fatty acid methyl esters were analyzed on a Varian 3600 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) with a temperature programmable injector and flame ionization detector. Separation of the fatty acid methyl esters was performed using an SP2560 fused silica capillary column (100 m x 0.25 mm (i.d.), with 0.25 µm film thickness) (Supelco, Bellefonte, PA, USA). Purified Helium (Praxair, Edmonton, Canada) was used as the carrier gas with a head pressure of 25 psi and a flow rate of 1 ml/min. The initial column temperature was set at 40 °C and held for 4 min, increased to 175 °C at 13 °C per min and held for 25 min, further increased to 215 °C at 4 °C per min and held for 23 min, and finally increased to 230 °C at 5 °C per min and held for 17.5 min. The initial injector temperature was 60 °C, held for 0.2 min, then increasing at 150 °C per min to 250 °C and held for 88 min. The detector temperature was set at 250 °C. Peak area was measured using the Scimadzu Class-VP chromatography data system (Shimadzu Scientific Instruments Inc, Columbia, MD, USA). Peaks were identified using Nu Chek Prep standards #85 and #411 (Nu Chek Prep, Elysian, MN, USA). Conjugated linoleic acids isomers were identified using standards from Matreya (Matreya, Inc., PA, USA). Detector response for individual fatty acids was verified using Nu Chek Prep standard #60 (Nu Chek Prep, Elysian, MN, USA). Each fatty acid was reported as a percentage of fatty acid methyl esters.

Milk for vitamin analysis was stored in opaque containers at –20 °C and vitamin extraction and preparation were performed away from direct sunlight. Vitamins E and A were extracted from milk using a method adapted from the procedure of Brubacher et al. (1985). Milk was thawed at 27 °C. A 5ml sample of the thawed milk was placed in a 50 ml tube. Twenty-five milliliters of methanol (containing 1.25 g ascorbic acid and 5 mg BHT) were added. After the addition of 5 ml of potassium hydroxide (50 % w/v in water) the tube was flushed with nitrogen and capped. The tube was inverted to mix contents, placed in an 80 °C water bath for 20 min with periodic agitation, and then cooled to 30 °C. The vitamin E and A were then extracted using heptane (Jensen and Nielsen, 1996; Jensen, 1994, Hidirogou, 1989). Five ml of heptane were added to the tube followed by vortexing for 1–2 min. The phases were then separated by centifugation at 2500 rpm for 5 min. The heptane layer was transferred to a 20 ml scintillation vial and evaporated under nitrogen at 40 °C. The extraction procedure was repeated with another 5 ml of heptane, which was transferred to the same scintillation vial for evaporation. After evaporation the residue was dissolved in 500 µl of acetone:chloroform v/v (3:7) and transferred to an HPLC vial for immediate analysis.

Vitamins were analyzed using a Waters 2690 HPLC system (Waters Associates Inc, Milford, MA, USA) fitted with a Supelcosil® RP LC-18 column (15 cm x 4.6 mm x 3 μm) (Supelco Canada Ltd, Oakville, ON, CA). The mobile phase was acetonitrile:methanol v/v (75:25) with a flow rate of 1 ml/min at a run time of 22 min. Vitamin E was determined using a Shimadzu RF-535 Fluorescence monitor (Shimadzu Scientific Instruments Inc, Columbia, MD, USA) with wavelength settings of 295 nm and 330 nm for excitation and emission respectively. Vitamin A was determined using a Waters 486 Tunable UV Absorbance detector (Waters Associates Inc, Milford, MA, USA) set at a wavelength of 325 nm. Peak area was measured using the Scimadzu Class-VP chromatography data system (Shimadzu Scientific Instruments Inc, Columbia, MD, USA). Vitamin E and A were quantified by comparison of peak areas to standard curves of α-tocopherol (Sigma-Aldrich, Inc., Mississauga, ON, Canada) and all-trans retinol (Sigma-Aldrich, Inc., Mississauga, ON, Canada) respectively.

3.2.3 Mammary Gland Biopsy and Analysis

A sample of mammary tissue was taken from 12 cows (six CON; six SAFF/M) during week nine using a method adapted from the procedure of Knight et al (1992). An intravenous catheter (14 g, 5.25 in) was first inserted into the jugular vein. Administration of Xylazine hydrochloride (10 mg/50 kg body weight) along with ketamine (2 mg/kg body weight) via the jugular catheter brought the animal into a state of deep sedation. With the cow laying on her side the legs were tied back to allow access to the udder. The animal was maintained in a state of sedation with an intravenous drip of glycerol guaiacolate (GGE) and ketamine (50 g GGE, 1000 mg ketamine, 1000 ml 50 % dextrose, and 900 ml sterile water). The drip rate was set at 20 ml/min and maintained for the duration of the surgery.

The udder was prepared for biopsy by shaving the area around the biopsy site and swabbing with iodine surgical scrub followed by 70 % v/v ethanol. A region in the basal portion of the udder that was free of major subcutaneous blood vessels was chosen for the biopsy site. The area was frozen with local anaesthetic using a line block subcutaneous injection of 10-30 ml of 2 % v/v lidocaine with epinephrine. An 8 cm incision was made in the skin. The capsule was exposed with blunt dissection followed by exposure of the mammary tissue. A 5-10 g piece of mammary tissue was excised using a scalpel. The tissue was immediately snap frozen in liquid nitrogen and stored at –80 °C until analysis. Bleeding was controlled through suturing and ligation using 3 metric catgut sutures. The surgical incision was closed using 4 metric catgut suture in the deep tissue and the skin incision was closed using #1 Vetafil in a Ford interlocking suture pattern. Once suturing was complete the drip was stopped and catheter removed. Recovery from sedation was rapid with the animal being able to return to its feet within 20 min of the cessation of the drip.

Expression of mammary stearoyl-CoA desaturase (SCD) was measured using the reverse transcritption (RT) and polymerase chain reaction (PCR) procedures following mRNA isolation from tissue. All reagents used were from Invitrogen™ life technologies (Invitrogen Canada Inc., Burlington, ON, Canada). Total RNA was isolated from pulverized mammary tissue with TRIzol® reagent and quantified by absorbance at 260 nm in a spectophotometer; only samples with a 260 nm : 280 nm ratio > 1.9 were used for further analysis. The reverse transcription (RT) and polymerase chain reactions (PCR) were carried out in a DNA thermocycler (PCR System 2400, Perkin Elmer, Mississauga, ON, Canada). First strand complementary DNA (cDNA) was synthesized from 2 µg of total RNA in a 20 µl reaction volume with a final concentration of 25 ng Oligo $(dT)_{12\text{-}18}$ primer, 0.5 m*M* dNTP mix, 4 µl of 5x first strand buffer, 0.01 *M* dithiothreitol, 2 U of RNaseOUT™, and 1 U of Supersript™ II RNase H^- reverse transcriptase. The reaction was carried out at 42 °C for 50 min, and 70 °C for 15 min. Aliquots of 2 µl of the first strand cDNA reaction were amplified in a 50 µl reaction volume containing a final concentration of 5 µl of 10x PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP mix, 2 U recombinant *Taq* DNA polymerase, and 0.4 µ*M* of each primer. Following an initial denaturation at 94 °C for 3 min, PCR was performed for a variable number of cycles (Table 3.3) of denaturation at 94 °C for 1 min, specific annealing temperature (Table 3.3) for 1 min, extension at 72 °C for 2 min, and a final extension of 72 °C for 10 min in the last cycle. The PCR products were electrophoresed on a 2 % agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide, the images captured with Gel Doc 1000 system, and the PCR product density analyzed with Molecular Analyst® software (Bio-Rad Laboratories, Mississauga, ON, Canada).

For each tissue sample, negative controls without reverse transcriptase yielded no amplification confirming that genomic DNA was not amplified (data not shown). Further, the primer pairs for SCD were designed to be located on different exons as a precaution against amplification of genomic DNA. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control (Delbecchi et al., 2001). In order to determine the optimal number of amplification cycles, cDNA samples were amplified from 12 to 30 cycles for GAPDH, and from 18 to 36 cycles for SCD. The optical density of GAPDH and SCD PCR products at each cycle number were plotted and the optimal cycle number was chosen from the linear range, as determined by a linear regression equation (Figure 3.1, 3.2) (Prism GraphPad Software Inc., San Diego, California). To ensure that PCR was conducted in the linear range of amplification, cDNA samples were amplified for 28 cycles for SCD and 24 cycles for GAPDH. Samples were analyzed in duplicate, PCR products were quantified as detailed above, and results expressed as the ratio of SCD to GAPDH.

3.2.4 Statistical Analysis

Dry matter intake, body weight, body condition score, and all milk data were analyzed statistically as a randomized complete block design with a repeated measures using the MIXED procedure of SAS version 8:3 (SAS Institute, Cary, NC). Cow within treatment was the experimental unit with week of sampling as the repeated measure. Treatment, week, block, and treatment by week interaction were fixed effects and cow was a random effect. The Kenward-Roger option was used to estimate denominator degrees of freedom. The variance-covariance matrix structure was chosen for each statistical model in a process wherein the best fit was chosen based on the Schwarz's Bayesian Criterion. Least square means were estimated and separated using the *pdiff* option when fixed effects were significant ($P<0.05$). Mammary RNA data was analyzed by ANOVA using the MIXED procedure of SAS.

3.3 Results and Discussion

3.3.1 Dry Matter Intake, Body Weight, and Body Condition Score

The effects of dietary treatment on dry matter intake (DMI), body weight, and body condition score are presented in Table 3.4 and Figures 3.3 to 3.5. Dry matter intake between treatments was not significantly different during the treatment period (Table 3.4, Figure 3.3). A significant week effect was observed for DMI ($P<0.0001$) as all groups decreased DMI over the treatment period (Figure 3.3). This was not unexpected. The animals began the treatment period at an average of 128 ± 47 DIM and by this point in the lactation cycle, cows have already past their peak DMI and milk production. The treatment by week interaction was not significant although the drop in DMI did appear to be more pronounced for the diets containing monensin compared with control (Figure 3.3). This effect of monensin was more clearly highlighted when considering DMI as a percentage of body weight. On this basis, DMI was lower for SAFF/M and SAFF/ME compared to CTL whereas the same treatments without monensin (SAFF and SAFF/E) were not significantly different from CTL (Table 3.4). This effect of monensin on DMI has been demonstrated numerous times before (Van der Werf et al., 1998, Sauer et al., 1998, Ramansin et al., 1997, Schelling, 1984) and is part of the reason for improved feed efficiency observed with monensin feeding in cattle. Dry matter intake as a percentage of body weight was also lower for FLAX/E compared to SAFF/E and CTL. The fact that the flaxseed treatment was lower than CTL but the safflower (without monensin) treatments were not might be explained by the difference in fatty acid composition. Safflower is particularly high in 18:2 n-6 (76%) whereas flaxseed oil is high in 18:3 n-3 (41.7%) with lower levels of 18:2 n-6 (21.3%) (Table 3.2). The large amount of 18:3 n-3 in the flaxseed treatment may have had a negative effect on cellulytic bacteria, decreasing fiber digestion, and hence lowering DMI through a rumen-fill effect (Palmquist, 1988). Another possibility is that 18:3 n-3 exerted a greater satiety effect than 18:2 n-3 through the stimulation of cholecystokinin at the small intestine (Allen, 2000).

Body weight and BCS were not significantly different between treatments (Table 3.4). However, the effect of week was significant as both body weight and BCS slowly increased between week 0 and week 9 for all groups (Figures 3.4, 3.5). This reflects a state of positive energy balance that is normal for mid-lactation cows.

3.3.2 Milk Production and Composition

Milk yield and milk composition is shown in Table 3.5. Milk yield was not significantly different between treatments although the effect of week was significant as yield declined for all groups during the treatment period (Figure 3.6). Contrary to milk yield, 4% fat-corrected-milk (FCM) yield was significant for treatment, week, and treatment by week interaction (*P*=0.037, *P*<0.0001, *P*=0.006, respectively) (Figure 3.7). The estimate of FCM can be more revealing than the simple milk yield in that it takes into account differences in fat percent and "corrects" yield accordingly. Fat-corrected-milk was not significantly different between groups at week 0. However, with the progression of the treatment period the effect of treatment on FCM yield became more apparent (Figure 3.7). Compared to CTL, the FCM yield was significantly lower for SAFF, SAFF/M, SAFF/ME, FLAX/E, and numerically lower for SAFF/E. This drop in FCM is explained by the depression in fat percent observed with safflower and flaxseed feeding compared to CTL.

All treatments except CTL showed a significant drop in milk fat percent between week 0 and week 2 (Figure 3.8). The addition of polyunsaturated fatty acids (PUFA) to the diet is commonly found to cause a depression in milk fat percentage (Bauman and Griinari, 2001). This depression is believed to be caused by rumen synthesized trans fatty acids, which inhibit milk fat synthesis in the mammary gland. The addition of vitamin E appeared to partially protect against this depression in milk fat (Figure 3.8). In fact, oilseed treatments that contained supplementary vitamin E (SAFF/E, SAFF/ME, FLAX/E) were not significantly different from CTL in milk fat percent, whereas oilseed treatments without vitamin E (SAFF, SAFF/M) had an overall significantly lower milk fat percent (Table 3.5). Other laboratories have observed a similar effect of vitamin E on bovine milk fat (Charmley and Nicholson, 1993; Charmley and Nicholson, 1994; Focant et al., 1998). The decrease in milk fat percent was also reflected in a decreased fat yield for all oilseed treatments (except SAFF/E) compared to CTL. The reason for this effect of vitamin E on milk fat percent is uncertain. In contrast to the effect of diet on milk fat, there was no significant difference in yield or concentration of milk lactose and protein during the treatment period (Table 3.5).

The vitamin E (α-tocopherol) content of milk was significantly higher for SAFF/E and SAFF/ME compared to all other treatments (Table 3.5, Figure 3.9). The supplementation of vitamin E was expected to increase the level of vitamin E in the milk even though the transfer efficiency from diet to milk is known to be relatively low in cows (Hidiroglou, 1989, Focant et al., 1998). It is not certain why the addition of vitamin E to the diet containing flaxseed oil did not increase vitamin E in milk to a similar extent as SAFF/E and SAFF/ME, even though this diet had levels of vitamin E very similar to

SAFF/E and SAFF/ME. There was also a significant week effect so that the increase in milk vitamin E was greater with the progression of the treatment period for SAFF/E and SAFF/ME (Figure 3.9). A similar yet less clear pattern was also true for SAFF, SAFF/M, and FLAX/E so that by week 8 the vitamin E concentration in these three treatments was significantly greater than CTL. This might be expected as safflower and flaxseed oils naturally contain vitamin E, which might be expected to increase the level of vitamin E in milk.

Banni et al. (1999) showed that feeding CLA to rats caused an increase in the amount of vitamin A in the mammary tissue and suggested that CLA may enhance the uptake and/or retention of CLA in the mammary gland. If this was also true in dairy cows it is possible that a greater retention of vitamin A in the mammary gland would lead to more vitamin A in the milk. In the present study, dietary treatment appeared to have a small effect on milk vitamin A concentration with significant treatment and week effects, and a strong trend (P=0.08) for treatment by week interaction (Figure 3.10). The SAFF, SAFF/E, SAFF/M, and SAFF/ME treatments each had higher milk vitamin A levels at week 8 compared to their respective week 0 values, whereas the vitamin A was not different between week 0 and 8 for CTL or FLAX/E. The overall treatment effect, independent of week, showed a small but significantly higher value for SAFF/ME compared to CTL and FLAX/E (Table 3.5). It is possible that these higher vitamin A concentrations observed with the safflower treatments were caused by the higher CLA produced by feeding safflower diets (see section 3.3.3).

3.3.3 Milk Fatty Acid Composition

The fatty acid compositions of the safflower and flaxseed oils are presented in Table 3.2. These oils were chosen for their high content of polyunsaturated fatty acids. Safflower is particularly high in 18:2 n-6 (76%) whereas flaxseed oil is high in 18:3 n-3 (41.7%) with lower levels of 18:2 n-6 (21.3%). In the rumen 18:2 n-6 can be converted to CLA and trans-11 18:1, whereas 18:3 n-3 appears to be converted to trans-11 18:1 but not CLA. Since the trans-11 18:1 can be converted to CLA in the mammary gland feeding oils high in 18:2 n-6 or 18:3 n-3 could potentially increase the level of CLA in milk.

The effect of dietary treatment on milk fatty acid composition is shown in Table 3.6 and Figures 3.11 to 3.16. The main characteristics of the safflower oil and flaxseed oil were to some extent reflected in the milk fatty acid composition. The addition of safflower or flaxseed oil significantly raised the level of 18:2 n-6 in the milk compared to CTL (Table 3.6, Figure 3.11), although this was much more pronounced for the safflower diets compared to the flaxseed. The addition of flaxseed to the diet increased the level of 18:3 n-3 in milk compared to CTL or safflower treatments (Table 3.6, Figure 3.12). Although a significant increase in 18:2 n-6 and 18:3 n-3 was observed in milk, the overall increase of these fatty acids in milk was relatively low, most likely because of extensive biohydrogenation in the rumen.

The rumen biohydrogenation process produces CLA and trans-11 18:1 as intermediate products. Moreover, as noted earlier the trans-11 18:1 can be converted to cis-9, trans-11 CLA in the mammary gland. The level of both CLA and total trans 18:1 fatty acids were significantly increased in milk with all safflower and flaxseed treatments compared to CTL (Table 3.6, Figures 3.13 and 3.14). Generally, the safflower oil treatments were more effective than flaxseed oil at increasing the level of CLA in milk (Table 3.6). The most likely explanation for this is that the predominant fatty acid in flaxseed, 18:3 n-6, produces trans-11 18:1 in the rumen but no CLA. Therefore, the increase in milk CLA with flaxseed feeding would be expected to come to a large extent from mammary desaturation of trans-11 18:1. Flaxseed feeding also increased the concentration of trans-11, cis-15 18:2 in milk compared to all other treatments. The rumen biohydrogenation of 18:3 n-3 is known to produce trans-11, cis-15 18:2, which is then converted to trans-11 18:1. The increase in trans-11, cis-15 18:2 in milk also helps explain the significantly lower trans 18:1 in milk for FLAX/E compared to the safflower treatments. It would be of interest to test the biological properties of this fatty acid as a dietary component in bovine milk considering that the FLAX/E treatment increased it to almost 3% of milk fatty acid methyl esters.

Within the safflower treatments, the two diets containing monensin (SAFF/M and SAFF/ME) had numerically higher levels of CLA in milk compared to the two diets without monensin (SAFF and SAFF/E) (Table 3.5). This difference was significant for SAFF/E compared to SAFF/M or SAFF/ME but was not significant for SAFF compared to SAFF/M or SAFF/ME. In the previous study, the difference between SAFF and SAFF/M was found to be statistically significant. The lack of significance for the SAFF-SAFF/M comparison in this study might be explained by the amount of variation in the SAFF/M results. In particular, two cows in this group appeared to have levels of CLA substantially below that of the rest of the SAFF/M group. An interesting observation was that individual animals with cis-9, trans-11 CLA levels that were noticeably low for their respective treatments also appeared to have noticeably higher levels of trans-10, cis-12

CLA. If this is in fact an accurate observation it might provide a clue that would help explain the variation between animals in their response to the dietary treatments. A higher level of trans-10, cis-12 at the expense of cis-9, trans-11 would suggest the rumen biohydrogenation pathways were directed towards trans-10 isomers instead of trans-11, possibly because of differences in bacterial populations among animals. This would result in less rumen synthesis of cis-9, trans-11 CLA, as well as less trans-11 18:1 for mammary cis-9, trans-11 synthesis. If differences in rumen bacterial populations do explain a large part of the variation in milk CLA, it would be interesting to test the hypothesis that inoculating pre-ruminant calves with "trans-11 producing" bacteria (as opposed to trans-10) would ensure that the animals have the "correct" bacterial populations for producing CLA-enriched milk in later life. This is an interesting speculation that is worthy of further study. The effect of monensin on milk fatty acid composition was more clearly observed in trans 18:1. The SAFF/M and SAFF/ME groups had significantly higher trans 18:1 in the milk compared to the SAFF and SAFF/E groups (Table 3.6). This might suggest that monensin was affecting the rumen bacterial population in a way that inhibited the conversion of trans 18:1 to 18:0. If this were the case, the level of 18:0 might be expected to be lower in the safflower treatments with monensin compared to safflower treatments without monensin. This in fact was the case and a strong trend towards lower 18:0 was observed for SAFF/M compared to SAFF (P=0.056), and SAFF/ME compared to SAFF/E (P=0.060). The higher level of milk trans 18:1 with monensin inclusion is also consistent with the higher cis-9, trans-11 observed for safflower/monensin treatments. An important question regarding monensin was whether it would continue to have an effect beyond two weeks. As can be seen from Figures 3.13 and 3.14, the effect of monensin on CLA and trans 18:1 persisted across the two-month period.

It has already been noted that the vitamin E partially prevented the depression in milk fat compared to the same treatments without vitamin E. The reason for this may be due to an effect on the rumen biohydrogenation pathways resulting in a change in the types of trans fatty acids synthesized, or in the overall quantity of trans fatty acids produced. The results of Table 3.6 provide evidence at least for the latter theory. The milk content of trans 18:1 is numerically lower for SAFF/E compared to SAFF, and significantly lower for SAFF/ME compared to SAFF/M. This reduction in trans 18:1 isomers may partially explain the alleviation in milk fat depression observed with vitamin E feeding in this study. An effect of vitamin E on trans 18:1 production in the rumen could decrease milk CLA content either through a reduction in the amount of trans-11 18:1 available for CLA synthesis in the mammary gland, or by a dilution of milk CLA because of the higher fat yield. However, since the values for milk CLA did not demonstrate the same pattern between treatments as trans 18:1, it is difficult to draw any firm conclusions regarding the effect of vitamin E on CLA from this trial.

The addition of safflower oil and flaxseed reduced the level of 16:0 and 14:0 in the milk by on average 40.1 % and 28.1 % respectively (Table 3.6; Figures 3.15 and 3.16). This is very similar to what was observed in the previous feeding study (Chapter 2). Since 16:0 and 14:0 potentially raise blood cholesterol when consumed in the diet, the large decrease in their concentration observed in this study is an additional benefit (Noakes et al., 1996). The concentration of the short to medium-chain fatty acids (4:0-

15:0) in milk were also reduced as a result of safflower and flaxseed feeding as is typically observed when the dairy diet is supplemented with fats and oils.

3.3.4 Mammary gland mRNA expression of Stearoyl-CoA Desaturase

Many studies, including the present one, have observed noticeable variation in CLA concentrations between cows fed the same diet (Jiang et al., 1996; Kelly et al., 1998; Lawless et al, 1998). Since a large proportion of milk CLA is synthesized in the mammary gland through the action of stearoyl-CoA desaturase (SCD), it has been suggested that differences in the expression or function of this enzyme might explain some of the observed variation. Animal differences in SCD could be at a number of levels including, gene expression, differences in enzyme structure associated with gene polymorphisms or post-translational modifications, or in differences in downstream factors that affect interaction between enzyme and substrate (Peterson et al., 2002). One aim of the present study was to determine if a correlation existed between the cis-9, trans-11 to trans-11 18:1 ratio in milk and mammary expression of SCD.

If cis-9, trans-11 CLA is synthesized in the mammary gland from trans-11 18:1 then we would expect to see a relationship in the milk concentration of these two fatty acids. The relationship between trans-11 18:1 and cis-9, trans-11 CLA in this study for all cows at all time points is shown in Figure 3.17. A strong linear relationship was observed ($R^2=0.7694$, $P<0.0001$), as has been reported by other laboratories (Lawless et al, 1998; Chilliard et al., 2001; Peterson et al., 2002). Although this relationship would also be expected based on the formation of trans-11 18:1 from cis-9, trans-11 18:2 in the rumen, studies have demonstrated that it is based mostly on the synthesis of cis-9, trans-11 18:2

from trans-11 18:1 in the mammary gland (Griinari et al., 2000). A similar relationship was observed for the ratios of 16:0 to 16:1 (Figure 3.18) and 14:0 to 14:1 (Figure 3.19).

This study used the reverse transcription polymerase chain reaction (RT-PCR) semi-quantitative technique to quantify expression of SCD in mammary tissue. RT-PCR is a well-established technique and is more sensitive and flexible than other mRNA quantification techniques such as northern hybridization (Bustin, 2000). A significant linear relationship ($R^2=0.8573$, $P=0.0239$) was found between the cis-9, trans-11 CLA to trans-11 18:1 ratio and SCD mRNA (reported as a ratio of SCD mRNA to glyceraldehyde 3-phosphate dehydrogenase mRNA) for cows in the SAFF/M group (Figure 3.20). Cows with higher expression of SCD might be expected to convert a greater proportion of available trans-11 18:1 to CLA, resulting in a relatively higher concentration of CLA in milk. The results presented in Figure 3.20 agree with this hypothesis, adding support to the idea that variation in SCD expression explains some of the variation observed in CLA concentrations between animals on the same diet.

Expression of SCD was not different for SAFF/M compared to CTL (Figure 3.21). Diets rich in n-6 and n-3 polyunsaturated fatty acids (PUFA) have been shown to suppress expression of SCD in different tissues of mice and rats (Ntambi, 1999; Raclot and Oudart, 1999). Similarly, feeding fish oil (high in n-3 PUFA) decreased the expression of mammary SCD in dairy cows (Ahnadi et al., 1998). However, because of extensive rumen biohydrogenation of PUFA, it is likely that the level of n-6 and n-3 PUFA reaching the mammary gland in this study was relatively low, which might explain the lack of effect on SCD in SAFF/M cows, compared to CTL. Trans-10 fatty acids have also been shown to inhibit enzymes involved in milk fat synthesis, including acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase (Park et al., 2000; Piperova et al., 2000; Peterson et al., 2002). However, since the level of trans-10, cis-12 18:2 was only 0.09% in SAFF/M milk (Table 3.6), it would appear that the level of trans-10 fatty acids reaching the mammary gland was also very low.

3.4 Conclusion

This study evaluated the ability of various combinations of polyunsaturated oils (safflower or flaxseed) with monensin and/or vitamin E to increase the concentration of CLA in bovine milk. The results confirmed that the combination of safflower oil with monensin was effective at increasing milk fat CLA and that the effect of monensin persisted over a period of two months. The addition of vitamin E to the diet partially prevented the depression in milk fat associated with the addition of oils to the dairy diet. It also decreased the level of total trans 18:1 in milk, although the effect on milk CLA was less clear. The flaxseed/vitamin E combination increased milk CLA concentrations compared to CTL, although the increase was less than that observed for the safflower diets. This study found a linear relationship between the expression of mammary SCD and the cis-9, trans-11 CLA to trans-11 18:1 ratio suggesting that cows with a greater ability to convert trans-11 18:1 to CLA had higher expression of SCD. This would add support to the hypothesis that genetic variations in SCD contribute to the variation observed in milk CLA between animals on the same diet.

Table 3.1 Ingredient and chemical composition of experimental diets.

Item	Treatments[1]					
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/M/E	FLAX/E
	(% of DM)					
Ingredient composition						
Barley silage	26.30	26.29	26.28	26.30	26.29	26.28
Alfalfa silage	21.20	21.19	21.19	21.20	21.19	21.19
Alfalfa hay	12.50	12.49	12.49	12.50	12.49	12.49
Ground corn	14.70	12.99	12.99	12.50	12.04	12.99
Barley	14.44	9.58	9.29	9.50	9.40	9.29
Safflower oil	0	6.00	6.00	6.00	6.00	0
Flaxseed oil	0	0	0	0	0	6.00
Soybean meal	5.00	5.00	5.00	5.00	5.20	5.00
Canola meal	0	2.00	2.00	2.00	2.00	2.00
Corn gluten meal	2.00	2.00	2.00	2.00	2.00	2.00
Animal fat	1.50	0	0	0	0.10	0
Limestone	0.60	0.60	0.60	0.60	0.60	0.60
Dicalcium phosphate	0.55	0.65	0.65	0.65	0.65	0.65
Mineral salt[2]	0.40	0.40	0.40	0.40	0.40	0.40
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Magnesium oxide	0.28	0.28	0.28	0.28	0.28	0.28
Sodium bicarbonate	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin ADE[3]	0.08	0.08	0.08	0.08	0.08	0.08
Monensin[4]	0	0	0	0.54	0.54	0
Vitamin E[5]	0	0	0.30	0	0.30	0.30
Chemical composition						
DM, %	43.9	44.1	44.0	43.8	43.5	44.4
CP	17.2	16.7	17.0	16.9	16.9	17.5
Crude fat	6.50	10.49	9.04	9.02	8.89	9.78
NDF	43.7	43.8	45.2	46.7	46.3	45.3
ADF	26.8	28.3	28.5	28.9	28.8	27.9
Ash	10.3	10.2	10.0	10.1	10.4	10.1
NE_L[6], Mcal/Kg	1.71	1.88	1.87	1.87	1.87	1.87

[1] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E).

[2] Contained: salt (min 95%), iodine (150 mg/kg), cobalt (50 mg/kg), copper (3500 mg/kg), manganese (10,000 mg/kg), zinc (9,000 mg/kg), and selenium (75 mg/kg).

[3] Contained: vitamin A (min 10,000,000 IU/kg), vitamin D (min 1,000,000 IU/kg), vitamin E (min 10,000 IU/kg).

[4] Contained: monensin (4,400 mg/kg).

[5] Contained: vitamin E (50,000 mg/kg).

[6] Estimated from NRC (1989)

Table 3.2 Fatty acid composition of safflower oil and flaxseed oil

Fatty acid	Safflower oil	Flaxseed oil
16:0	6.7 ± 0.57	5.6 ± 0.19
18:0	2.3 ± 0.22	4.0 ± 0.40
18:1n-9	14.8 ± 0.86	23.1 ± 1.28
18:2n-6	76.0 ± 0.24	21.3 ± 2.31
18:3n-6	nd	41.7 ± 2.63
Other	0.3 ± 0.17	4.3 ± 1.33

nd = not detected; numbers represent averages ± standard deviation

Table 3.3. Primer pairs for RT-PCR amplification of each target gene, annealing temperature (AT), number of cycles of amplification, and length (base pairs, bp) of PCR products.

Target	Primers[1]	AT (°C)	Cycles	Length (bp)
SCD[2]	F: 5'-TCTATGACCCAACTTACCAGG -3'	55° C	28	648
	R: 5'-TATGGGCAGCACTATTCACCAGCCAG -3'			
GAPDH[3]	F: 5'-CTGGCAAAGTGGACATTGTCGCC-3'	65° C	24	572
	R: 5'-CTTGGCAGCGCCGGTAGAAGC-3'			

[1] F, R: Forward and Reverse primers.

[2] Stearoyl-CoA desaturase - Bovine–specific primer sequences: GI accession # 19851965

[3] Glyceraldehyde 3-phosphate dehydrogenase - Bovine–specific primer sequences: GI accession #: 2285902

Table 3.4 Dry matter intake (DMI), body weight, and body condition score (BCS) during the treatment period, independent of week.

	Treatments[1]						
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	Sem
DMI, kg/d	19.06	18.75	18.81	17.01	17.72	17.76	0.68
DMI (% body weight)	3.15^{a}	3.02^{ac}	3.10^{ad}	2.70^{b}	2.83^{bcd}	2.77^{bc}	0.11
Body weight, kg	605.4	623.8	611.3	633.2	632.58	647.3	14.55
BCS (1 to 5 scale)	2.92	3.00	2.94	2.93	3.06	3.07	0.09

[1] Diets were control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E).

[a,b,c] Values within a row with different letters are significantly different at $P<0.05$

Table 3.5 Milk yield and composition during the treatment period, independent of week.

	Treatments[1]						
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	Sem
Milk yield, kg/d	32.02	29.81	31.01	29.89	28.52	29.36	1.44
4% FCM[2]	29.85^{a}	24.34^{b}	27.65^{ab}	24.77^{b}	24.86^{b}	25.94^{b}	1.36
Lactose, %	4.60	4.60	4.63	4.56	4.53	4.62	0.05
Lactose yield, kg/d	1.45	1.33	1.43	1.36	1.26	1.33	0.07
Protein, %	3.04	3.06	3.13	2.98	3.18	3.12	0.08
Protein yield, kg/d	0.95	0.87	0.96	0.88	0.85	0.89	0.04
Fat, %	3.66^{a}	2.97^{bc}	3.26^{ab}	2.85^{b}	3.28^{ab}	3.30^{ac}	0.16
Fat yield, kg/d	1.15^{a}	0.85^{b}	1.02^{ab}	0.86^{b}	0.92^{b}	0.96^{b}	0.06
Vitamin E, µg/g milk	0.72^{a}	0.81^{a}	1.28^{b}	0.83^{a}	1.31^{b}	0.87^{a}	0.06
Vitamin A, µg/g milk	0.36^{a}	0.40^{ab}	0.40^{ab}	0.39^{ab}	0.42^{b}	0.36^{a}	0.02

[1] Diets were control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E).

[2] 4% FCM = (0.4 x Milk yield) + (15 x Fat yield)

[a,b,c] Values within a row with different letters are significantly different at $P<0.05$

Table 3.6 Milk fatty acid composition during the treatment period, independent of week.

	Treatment[1]						
Fatty Acid[2]	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	Sem
4:0	4.12^{a}	2.77^{b}	3.04^{bc}	2.81^{b}	2.93^{bc}	3.23^{c}	0.13
6:0	2.37^{a}	1.39^{b}	1.54^{b}	1.42^{b}	1.50^{b}	1.56^{b}	0.07
8:0	1.19^{a}	0.63^{b}	0.70^{b}	0.64^{b}	0.68^{b}	0.70^{b}	0.04
10:0	2.53^{a}	1.26^{b}	1.40^{b}	1.27^{b}	1.38^{b}	1.38^{b}	0.08
11:0	0.343^{a}	0.136^{b}	0.167^{cd}	0.140^{bd}	0.161^{bc}	0.185^{c}	0.01
12:0	2.87^{a}	1.53^{b}	1.67^{b}	1.55^{b}	1.64^{b}	1.64^{b}	0.09
13:0	0.20^{a}	0.10^{b}	0.13^{b}	0.11^{b}	0.12^{b}	0.12^{b}	0.01
14:0	11.64^{a}	8.10^{b}	8.48^{b}	8.32^{b}	8.44^{b}	8.48^{b}	0.24
14:1	0.95^{a}	0.57^{b}	0.62^{b}	0.54^{b}	0.57^{b}	0.60^{b}	0.05
15:0	1.08^{a}	0.74^{b}	0.76^{b}	0.78^{b}	0.78^{b}	0.79^{b}	0.02
16:0	30.60^{a}	18.70^{b}	18.35^{b}	17.99^{b}	18.71^{b}	17.87^{b}	0.54
16:1	1.53^{a}	0.96^{b}	0.95^{b}	0.90^{b}	0.96^{b}	0.91^{b}	0.05
18:0	9.76^{a}	11.43^{bc}	11.63^{bd}	10.30^{ac}	10.51^{acd}	11.08^{bc}	0.41
18:1 trans	2.63^{a}	14.25^{b}	13.10^{b}	18.30^{c}	16.26^{d}	8.87^{e}	0.58
18:1 n-9	19.26^{ab}	21.14^{bc}	21.79^{c}	18.67^{a}	19.48^{ab}	24.00^{d}	0.68
18:1 n-7	0.50	0.64	0.52	0.61	0.54	0.53	0.05
18:2 t-11, c-15	0.43^{a}	0.57^{a}	0.54^{a}	0.55^{a}	0.52^{a}	2.99^{b}	0.08
18:2 n-6	1.75^{a}	2.89^{b}	2.82^{b}	2.96^{b}	2.81^{b}	2.01^{c}	0.08
18:3 n-3	0.41^{a}	0.32^{b}	0.33^{b}	0.32^{b}	0.32^{b}	0.73^{c}	0.01
20:0	0.20^{a}	0.17^{a}	0.18^{a}	0.16^{a}	0.17^{a}	0.53^{b}	0.02
20:1 n12	0.17^{a}	0.13^{b}	0.13^{b}	0.12^{b}	0.12^{b}	0.18^{a}	0.01
20:1 n-9	0.07^{a}	0.09^{b}	0.08^{ab}	0.11^{c}	0.10^{bc}	0.24^{d}	0.01
CLA c-9, t-11[3]	0.68^{a}	4.12^{bc}	3.48^{bd}	4.55^{c}	4.75^{c}	2.80^{d}	0.31
CLA t-10, c-12	nd^{a}	0.04^{b}	0.06^{bc}	0.09^{c}	0.06^{bc}	nd^{a}	0.01
Other FA	4.70^{a}	7.28^{b}	7.47^{b}	6.78^{c}	6.43^{d}	8.51^{e}	0.12
Saturated	66.90^{a}	46.95^{b}	48.05^{b}	45.50^{b}	47.03^{b}	47.55^{b}	0.91
Unsaturated	27.44^{a}	45.20^{bd}	43.85^{b}	47.18^{d}	45.96^{cd}	43.33^{b}	0.90
Unsat:Sat	0.41^{a}	0.98^{bc}	0.93^{b}	1.04^{c}	0.99^{bc}	0.92^{b}	0.03

[1] Diets were control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E).

[2] Fatty acids are expressed as a percentage of fatty acid methyl esters

[3] The chromatography methods used were unable to separate the minor quantities of 7/9 and 8/10 CLA isomers from the main cis-9, trans-11 isomer.

[a,b,c] Values within a row with different letters are significantly different at $P<0.05$

Figure 3.1 – Optical density of stearoyl-CoA desaturase (SCD) cDNA products after 18 to 36 amplification cycles by polymerase chain reaction

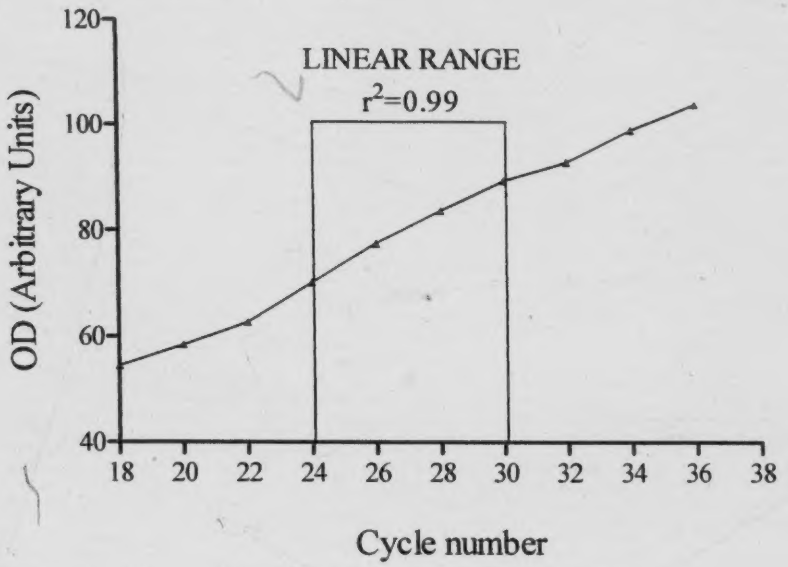


Figure 3.2 – Optical density of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA products after 12 to 28 amplification cycles by polymerase chain reaction

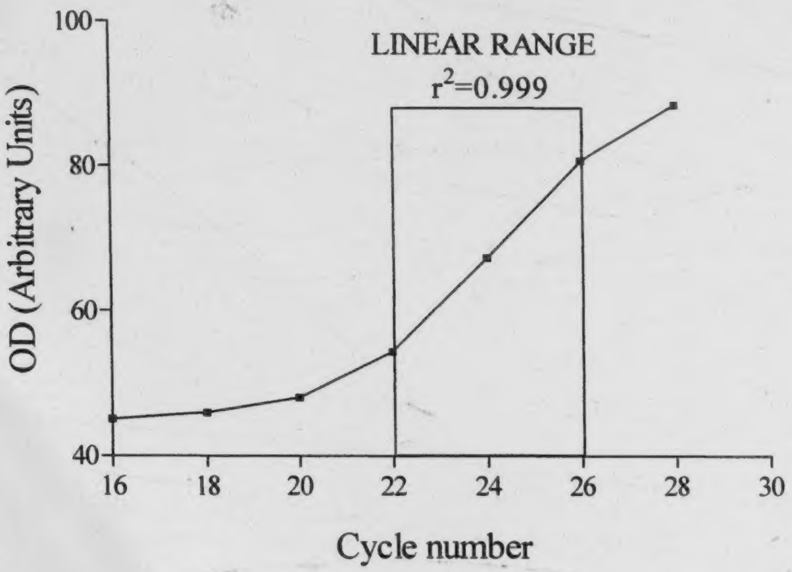
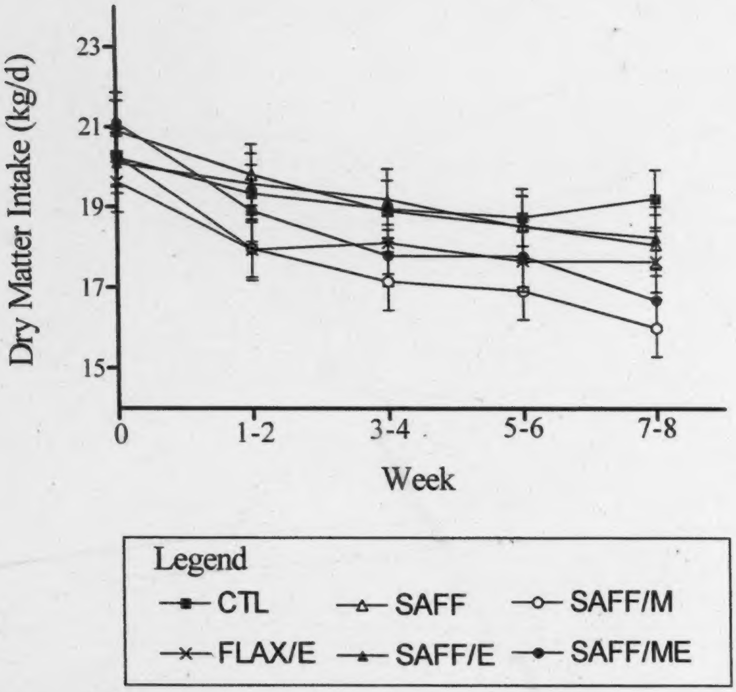


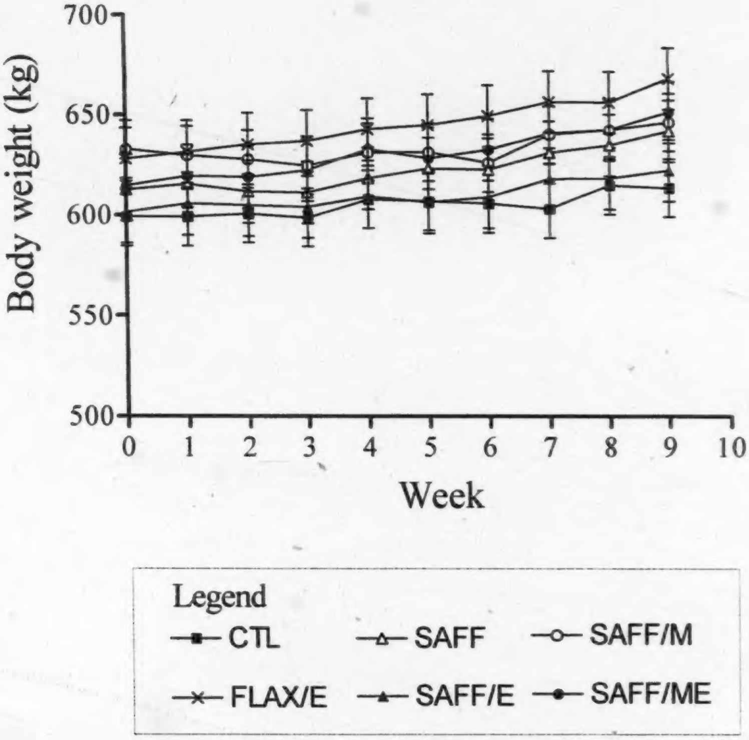
Figure 3.3 – Average biweekly dry matter intake of treatment groups[1,2]



[1] Dry matter intake at each indicated time interval represents the average dry matter intake for each group over that time period.

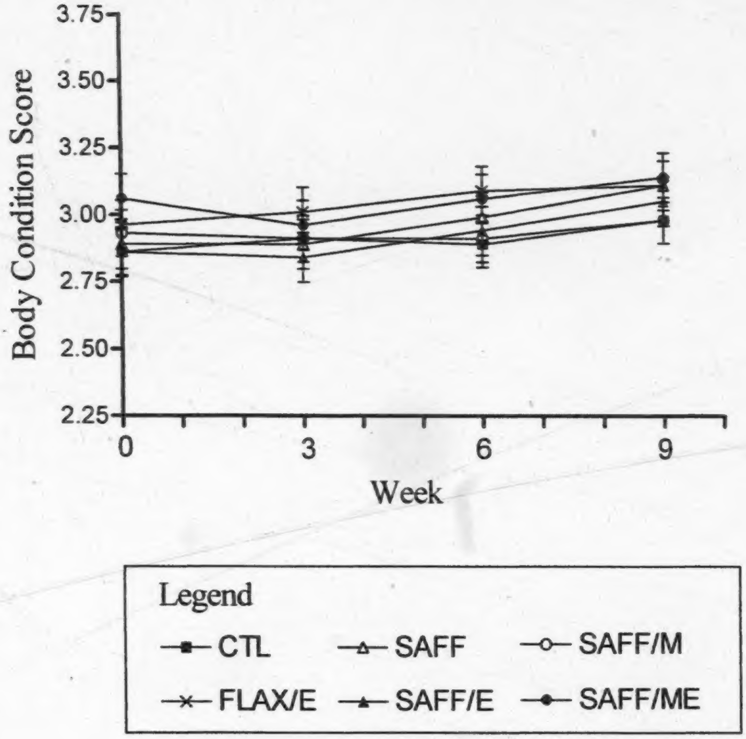
[2] Significant week effect (*P*<0.0001); treatment (excluding week 0 data) and treatment*week interaction not significant (*P*=0.21 and *P*=0.13 respectively).

Figure 3.4 – Average body weight of treatment groups per week[1]



[1] Significant week effect ($P<0.0001$); treatment (excluding week 0 data) and treatment*week interaction not significant ($P=0.34$ and $P=0.30$ respectively).

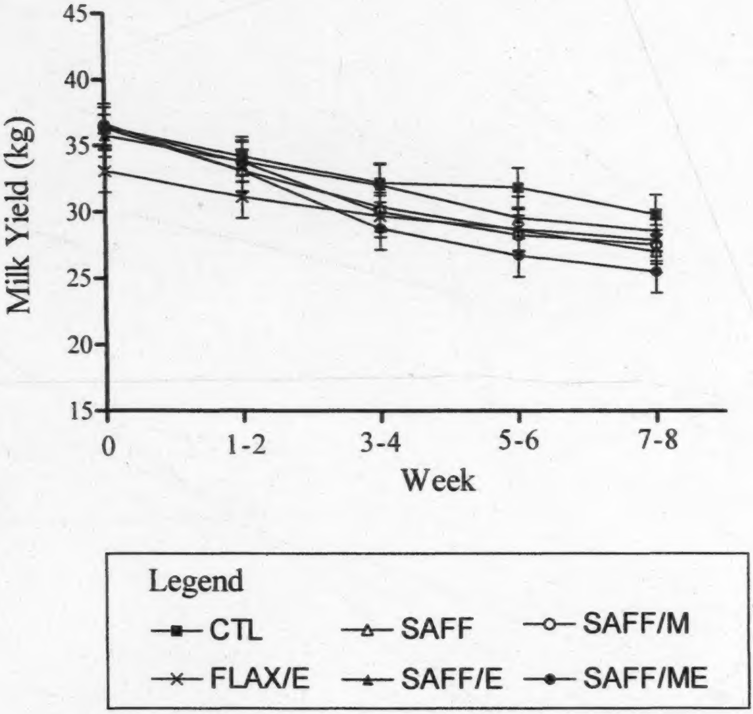
Figure 3.5 – Average body condition score of treatment groups at weeks 0, 3, 6, 9[1,2].



[1] Body condition score measured on a 5-point scale, one being extremely thin, five being obese.

[2] Significant week effect (*P*<0.0001); treatment (excluding week 0 data) and treatment*week interaction not significant (*P*=0.74 and *P*=0.35 respectively).

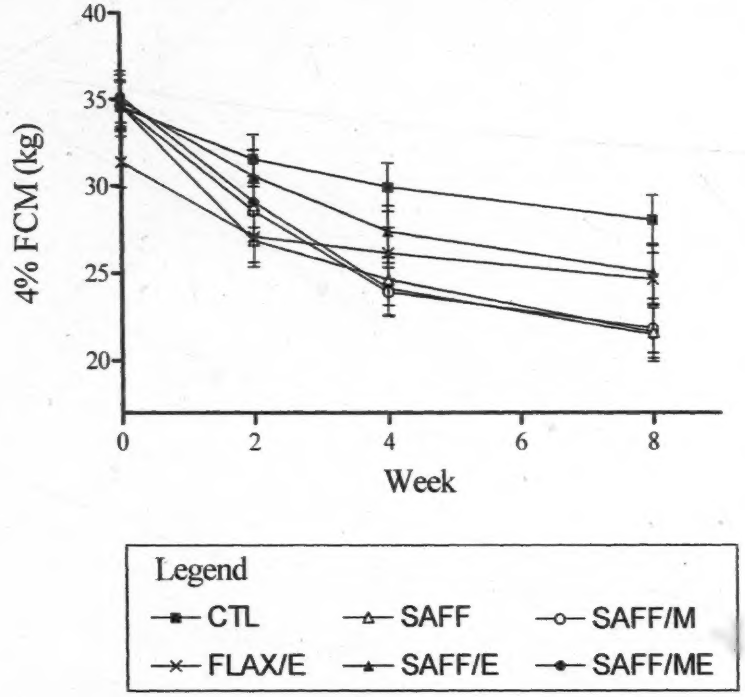
Figure 3.6 – Average biweekly milk yield of treatment groups[1,2]



[1] Milk yield at each indicated time interval represents the average milk yield for each group over that time period.

[2] Significant week effect (*P*<0.0001); treatment (excluding week 0 data) and treatment*week interaction not significant (*P*=0.58 and *P*=0.08 respectively).

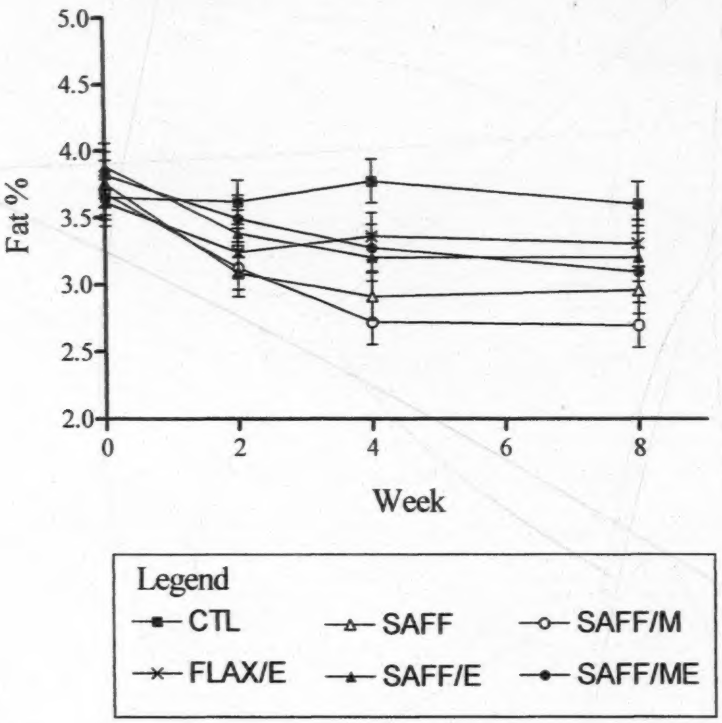
Figure 3.7 – Average biweekly 4%FCM of treatment groups[1,2]



[1] 4% FCM at each indicated time interval represents the average milk yield for each group over that time period.

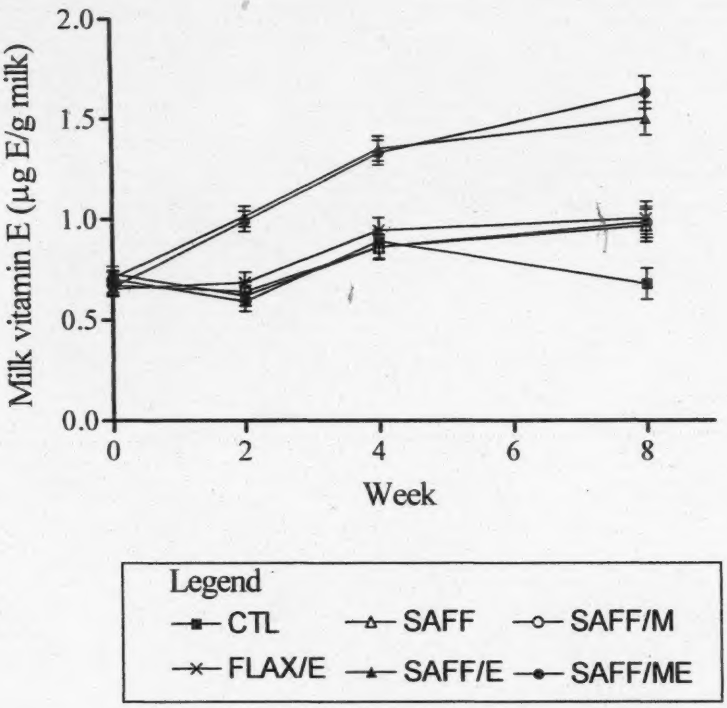
[2] Significant treatment (excluding week 0 data), week, and treatment*week interaction (*P*=0.037, *P*<0.0001, and *P*=0.006 respectively).

Figure 3.8 – Average milk fat % of treatment groups at weeks 0, 2, 4, 8[1].



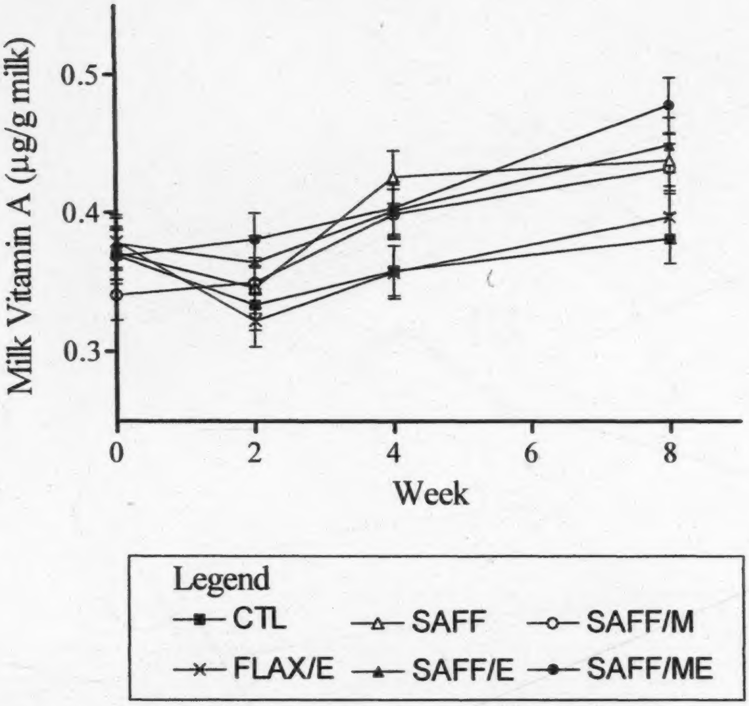
[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant (P=0.008, P<0.0001, P<0.0001 respectively)

Figure 3.9 – Average milk vitamin E (μg α-tocopherol/g milk) of treatment groups at weeks 0, 2, 4, 8[1].



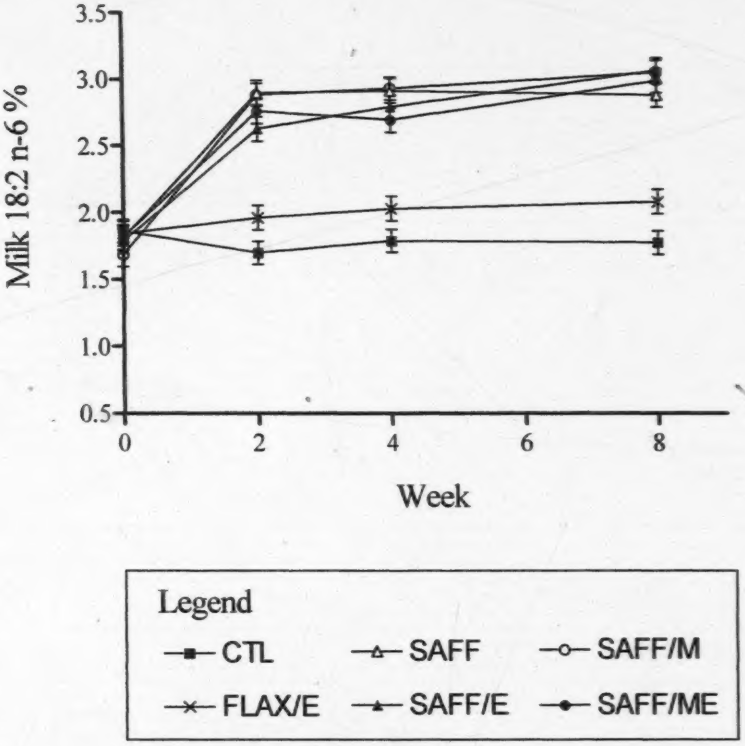
[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant (P<0.0001).

Figure 3.10 – Average milk vitamin A (μg/g milk) of treatment groups at weeks 0, 2, 4, 8[1].



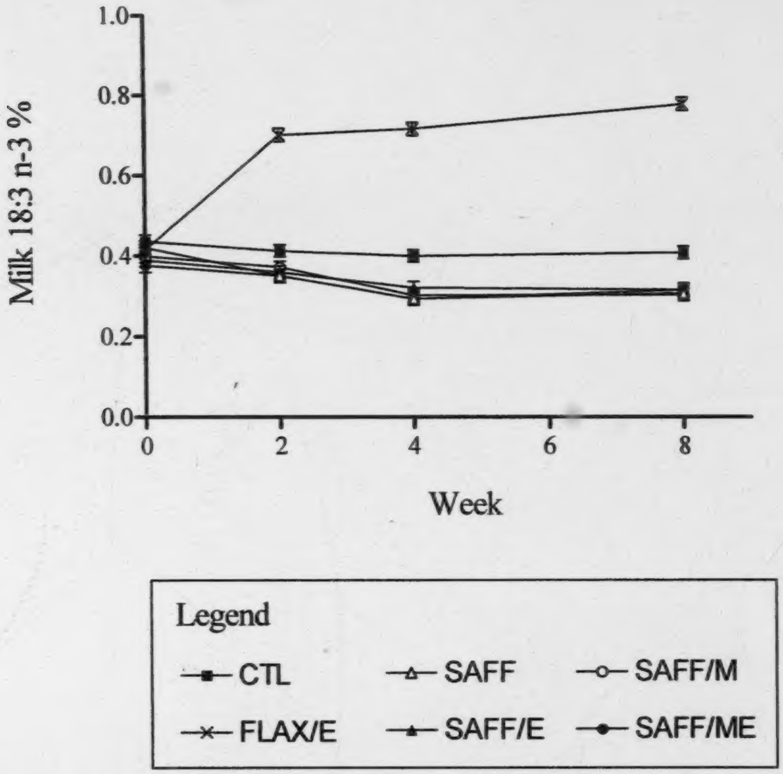
[1]Treatment (excluding week 0 data) and Week - significant (P=0.04 and P<0.0001, respectively). Treatment*Week interaction not significant (*P*=0.08).

Figure 3.11 – Average milk 18:2 n-6 % of treatment groups at weeks 0, 2, 4, 8[1].



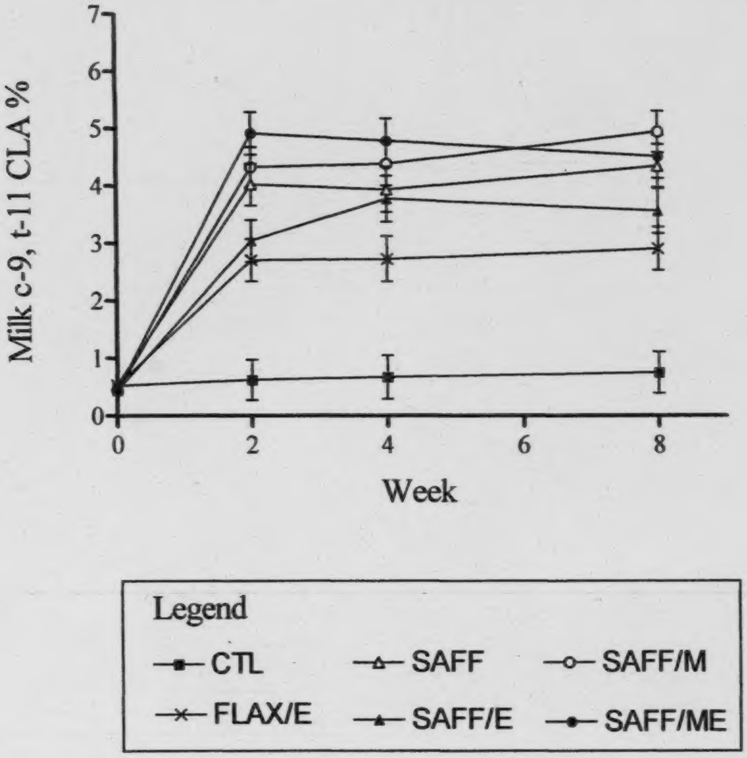
[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant ($P<0.001$, $P<0.0001$, $P<0.0001$ respectively)

Figure 3.12 – Average milk 18:3 n-3 % of treatment groups at weeks 0, 2, 4, 8[1].



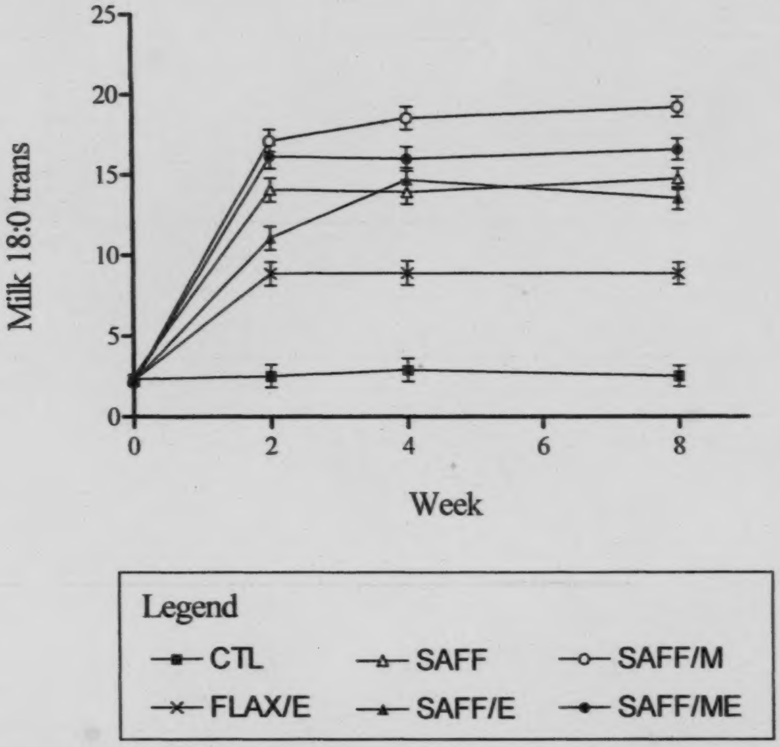
[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant ($P<0.001$, $P<0.0001$, $P<0.0001$ respectively)

Figure 3.13 – Average milk cis-9, trans-11 CLA % of treatment groups at weeks 0, 2, 4, 8[1].



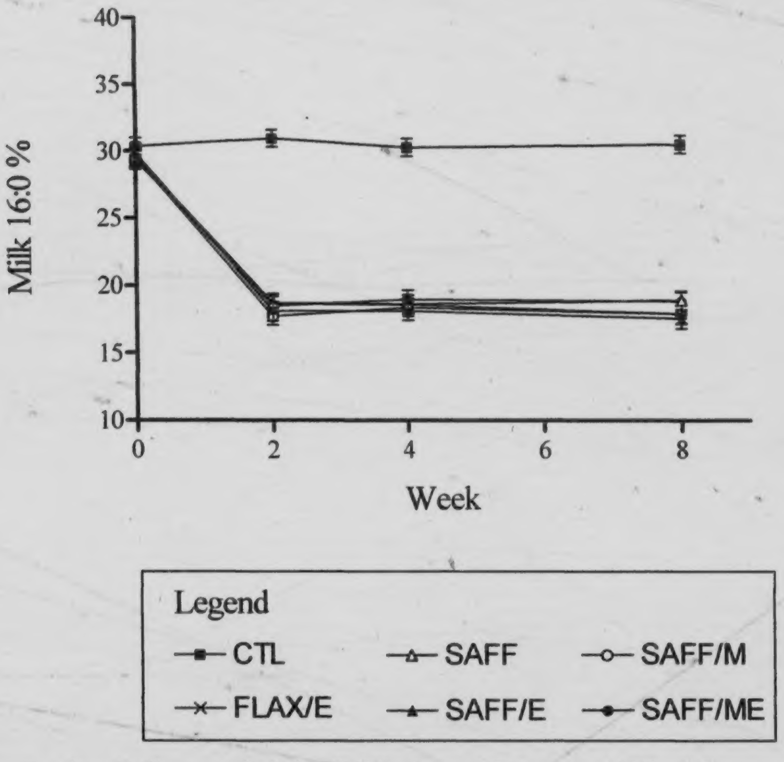
[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant (P<0.001, P<0.0001, P<0.0001 respectively)

Figure 3.14 – Average milk 18:1 trans % of treatment groups at weeks 0, 2, 4, 8[1].



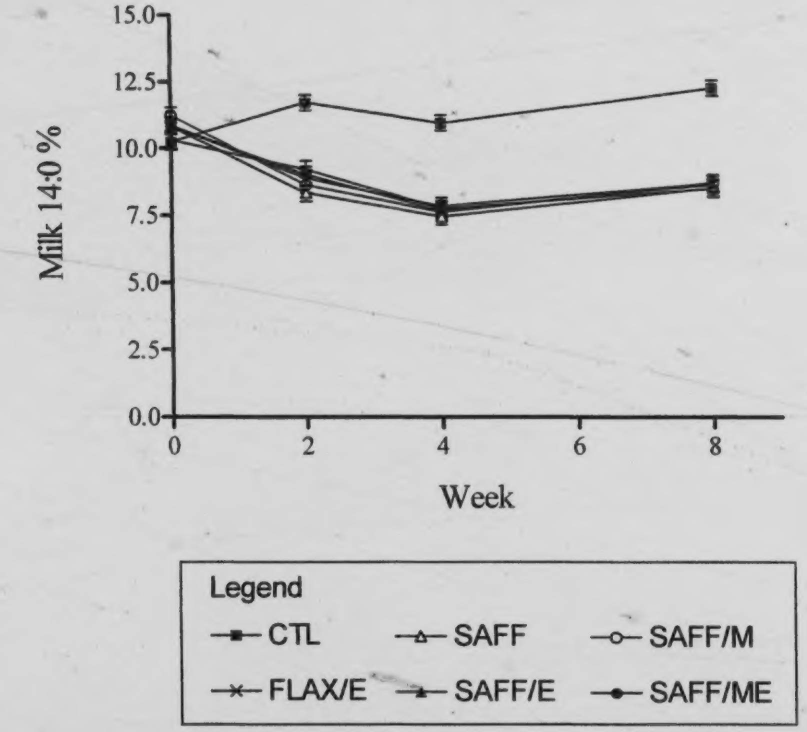
[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant (P<0.001, P<0.0001, P<0.0001 respectively)

Figure 3.15 – Average milk 16:0 % of treatment groups at weeks 0, 2, 4, 8[1].



[1] Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant ($P<0.001$, $P<0.0001$, $P<0.0001$ respectively)

Figure 3.16 – Average milk 14:0 % of treatment groups at weeks 0, 2, 4, 8[1].



[1]Treatment (excluding week 0 data), Week, and Treatment*Week interaction - significant ($P<0.001$, $P<0.0001$, $P<0.0001$ respectively)

Figure 3.17 - Relationship between trans 18:1 and c-9, t-11 CLA in milk

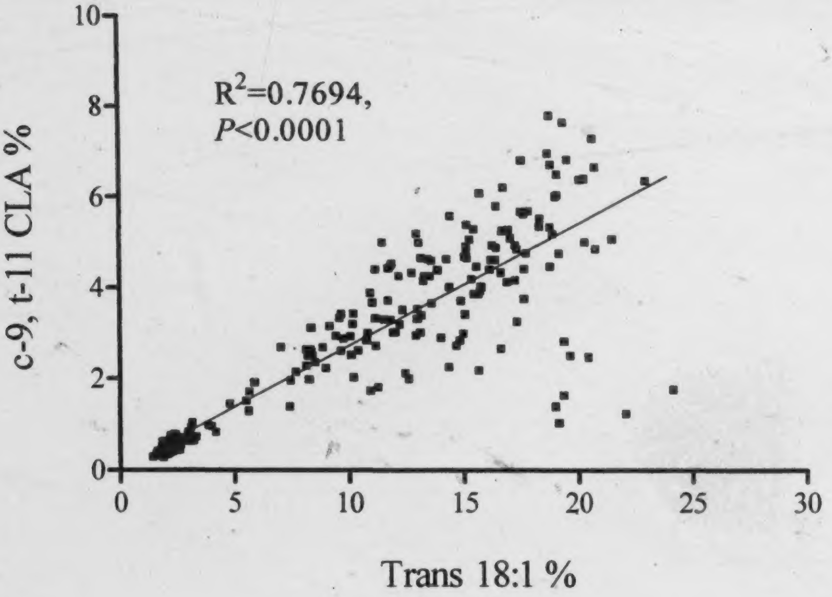


Figure 3.18 - Relationship between 16:0 and 16:1 in milk

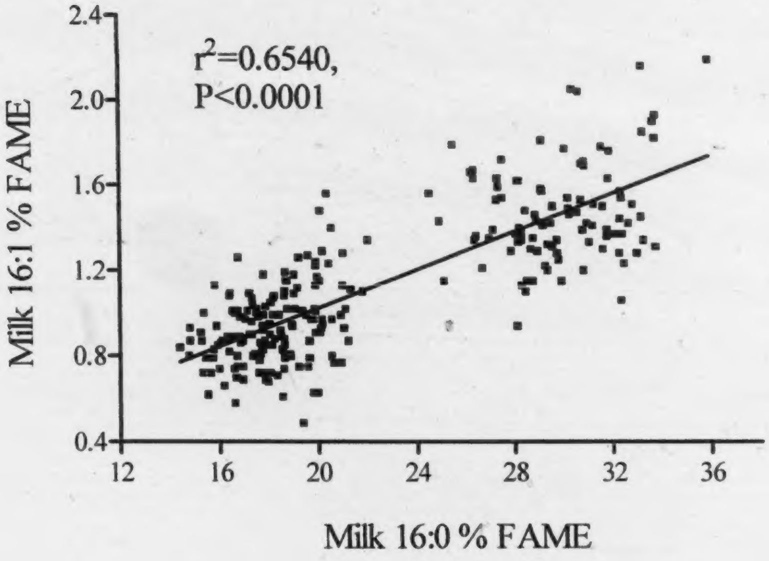


Figure 3.19 - Relationship between 14:0 and 14:1 in milk

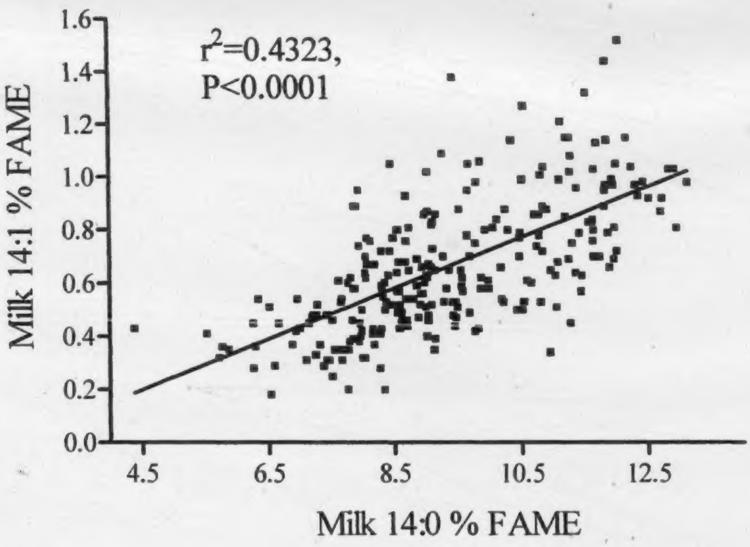


Figure 3.20 - Relationship between CLA:trans 18:1 in milk and SCD:GAPDH mRNA in mammary tissue of SAFF/M cows.

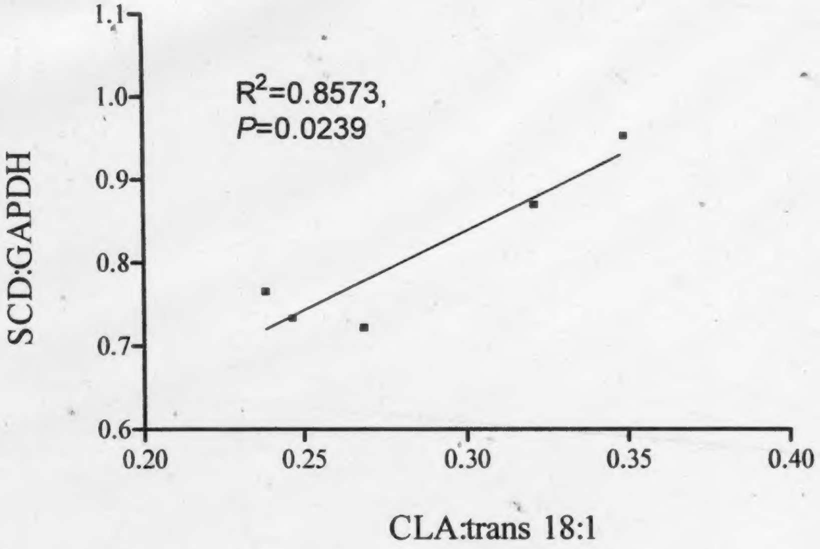
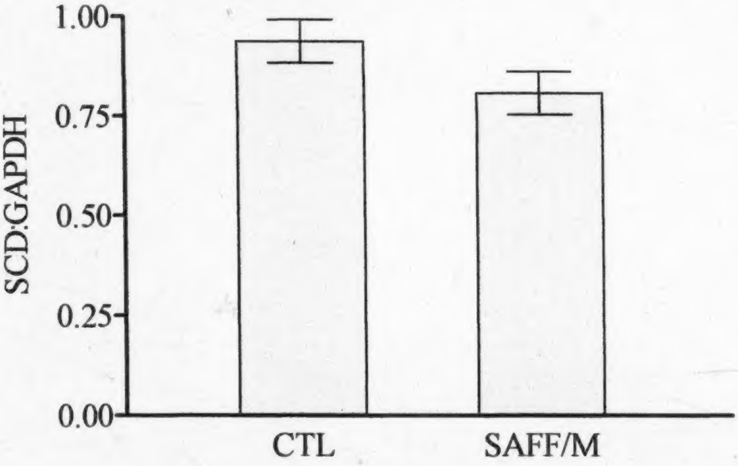


Figure 3.21 - Ratio of SCD to GAPDH in mammary tissue for CTL and SAFF/M.



Treatment effect not significant (*P*=0.13)

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Chapter 4 – Sensory evaluation of conjugated linoleic acid-enriched milk.

4.1 Introduction

Diets that increase CLA in bovine milk produce a number of other significant changes such as an increase in monounsaturated, polyunsaturated, and trans fatty acids, and a decrease in short-chain and total saturated fatty acids (Chapters 2 and 3). This raises the question for whether the changes in fatty acid composition have an effect on the organoleptic characteristics of the milk, and whether CLA-enriched milk will be an acceptable product to consumers.

This study was carried out in conjunction with a dairy production trial that evaluated the ability of dairy nutrition to increase the concentration of CLA in bovine milk (Chapter 3). A concern associated with elevating the concentration of unsaturated fatty acids in milk is the greater susceptibility to oxidation (Palmquist et al., 1993). Oxidation of double bonds produces hydroperoxides that result in the formation of volatile aldehydes and associated carbonyl compounds, giving the milk a distinctive off flavor (Charmley and Nicholson, 1994). However, vitamin E is a natural antioxidant capable of protecting milk fat against oxidation (Atwal et al., 1991; Nicholson and St-Laurent, 1991; Ashes et al., 1997; Focant et al., 1998). Vitamin E from the diet becomes incorporated into the milk fat globule membrane and acts as an electron sink to counter the activity of divalent cations such as copper and selenium, which catalyze oxidation in milk (Ashes et al., 1997). Some of the dietary treatments used in the study presented here included vitamin E in anticipation of problems with oxidation. The main objective of this study was to evaluate the organoleptic characteristics of CLA-enriched milk using trained sensory evaluation as well as an untrained consumer acceptability testing.

4.2 Materials and Methods

4.2.1 Animals and Treatments

Details of animals and diets used to obtain milk for sensory evaluation were presented in Chapter 3. Briefly, the treatments were: (1) Control diet (CTL); (2) Control diet including safflower oil supplemented at 6% of DM (SAFF); (3) Control diet including safflower oil supplemented at 6% of DM plus Vitamin E supplemented at 150 IU/kg DM (SAFF/E); (4) Control diet including safflower oil supplemented at 6% of DM plus monensin supplemented at 24 ppm of DM (SAFF/M); (5) Control diet including safflower oil supplemented at 6% of DM plus Vitamin E supplemented at 150 IU/kg DM plus monensin supplemented at 24 ppm of DM (SAFF/M/E); (6) Control diet including flaxseed oil supplemented at 6% of DM plus Vitamin E supplemented at 150 IU/kg DM (FLAX/E).

4.2.2 Milk Collection and Processing

Milk for trained sensory evaluation was collected on the third-last day and last day of weeks 0, 2, 4, and 8. On each of these days the total AM milk was collected from five cows in each treatment group. Milk taken from each cow was mixed by pouring back-and-forth between two large pales. Seven liters were then removed, placed in a closed container and immediately cooled on ice water. Milk from the five cows was pooled to give 35 liters per treatment. Milk was also collected at the same time and in the same manner from five cows that were fed the standard Dairy Research and Technology Centre (DRTC) dairy diet, which was similar to the experimental control. This milk was used as an identified reference control for the sensory evaluation panel. Milk was also collected on the last day of week nine from CTL, SAFF/M and SAFF/ME groups. This milk was used for consumer acceptability testing.

After collection the milk was taken immediately to the Alberta Agriculture, Food and Rural Development Food Processing Development Centre, Leduc, Alberta, Canada for processing and sensory evaluation. In order to avoid bias in the sensory evaluation process, the milk collection containers were identified only with color and number coding. The sensory manager, sensory technician and panelists were therefore blind to the identity of the milk, except for the identified reference control. Milk was standardized, pasteurized and homogenized prior to sensory analysis as follows. The milk was first separated using a bench-top type DD Westfalia separator (Westfalia-Surge Inc., Mississauga, ON) into cream and skim. Milk fat percent of the cream and skim portions was measured using the Babcock method (Martin, 1978). A portion of the cream was added back to the skim to produce milk with three-percent fat. The milk was then batch-pasteurized at 68 °C for 2.5 minutes using a Lee 20 Liter agitated steam-kettle Model D9MT (Lee Process Systems and Equipment, Philipsburg, Pennsylvania). The pasteurized milk was homogenized in a Gaulin M3 two-stage homogenizer (Gaulin Corporation, Everett, MA, USA) at pressures of 2500 psi and 500 psi for the first and second stages, respectively. The processed milk was collected in four-liter plastic containers and immediately refrigerated. The alkaline-phosphatase test was carried out on each batch of processed milk to monitor pasteurization effectiveness.

Samples were stored at 2 °C in a commercial cooler in order to preserve freshness until sensory evaluation on the following the day. Samples for shelf life testing were stored to simulate a home refrigerator. These were stored at 4 °C for five days starting from the day of processing. The identified reference control presented with the shelf life samples was processed the same day as the non-shelf life samples and stored for the five days at 2°C in a commercial cooler in order to preserve freshness.

4.2.3 Sensory Evaluation

The sensory evaluation was carried out under contract by Karin Erin at the Alberta Agriculture, Food and Rural Development Food Processing Development Centre, Leduc, Alberta, Canada. The techniques employed for both trained and untrained panelist screening, training, and sensory testing were based on the methods of Cross et al. (1978), Neilgarrd et al. (1991), and Ogden (1992).

Potential panelists for the trained sensory evaluation were first pre-screened based on milk consumption. Only those that ordinarily drink milk were selected. The ability of these selected individuals to detect differences in the organoleptic characteristics of milk was determined during four one-hour training sessions using 16 triangle tests. The purpose of these training sessions was to eliminate unsuitable panelists. Nine panelists (five females, four males; average age 50, range 34-70) were chosen and underwent 13 formal one-hour training sessions prior to the start of the study. During the course of the study four additional refresher-training sessions were completed.

During the training and testing each panelist was provided with non-trial identified reference milk. The non-trial reference milk was obtained from five cows from the University of Alberta herd that were fed the standard DRTC dairy diet. This milk provided a reference or benchmark for each sensory characteristic. The panelists were unaware that one of the six "treatment" samples was an experimental control (CTL), allowing unbiased judgment to be maintained. During the training and testing each panelist was also provided with five reference samples of common off-flavors in milk: malty, bitter, sour, flat, and photo-oxidized. The off-flavor references were prepared as follows: The malty reference was prepared by mixing 15 g of All Bran cereal into 100 g of milk, allowing it to sit at room temperature for 30 minutes, and then filtering to remove cereal particles, thereby obtaining the malty stock solution. Malty references for the panelists each session were 3.73% malty stock solution in control milk (or 22 ml of malty stock solution in 590 ml of control milk). The bitter reference was prepared by mixing 4 g of caffeine powder into 200 ml of water to obtain a 2% bitter stock solution. Bitter references for the panelists each session were 0.03% bitter stock solution in control milk (or 3 ml of bitter stock solution in 200 ml of control milk). The sour reference was prepared by mixing 10 g of lactic acid powder into 200 ml of water to obtain a 5% sour stock solution. Sour references for the panelists each session were 0.3% sour stock solution in control milk (or 12 ml of sour stock solution in 200 ml of control milk). The flat reference was prepared by mixing one part water to two parts control milk. The photo-oxidized reference was prepared by exposing 350 ml of control milk to ultraviolet light for 15.5 h. This 15.5-h photo-oxidized milk was then diluted with non photo-oxidized control milk in a 1:1 ratio (producing diluted photo-oxidized milk). Finally, the photo-oxidized references for the panelists in each session were prepared by mixing 2 parts of diluted photo-oxidized milk with one part of control non photo-oxidized milk.

Samples for sensory evaluation were portioned between 8:00 am to 9:30 am on the morning of testing. After portioning, samples were placed in a refrigerator at 4 °C, until removal and placement in an incubator (set at 18 °C) one hour before the scheduled panel time. Panel times were set at 11:00 am and 1:30 pm. All samples were presented in one ounce portion cups, fitted with a plastic lid. The serving temperature of the milk was approximately 17 °C. Panelists received 10 ml of each reference sample per session. Additional reference samples were provided if required. Each panelist received two identified reference control samples (10 ml each) at the beginning of each session. One additional reference control was presented with the fifth of seven samples. Additional reference control samples were provided if required. Each panelist received two coded samples (10 mL each) of each treatment at one sitting. Additional coded treatment samples were provided if requested. In addition, each panelist received two hidden coded reference control samples (10 ml each) during each session. Each panelist received the coded treatment samples in a different order. Three unsalted soda crackers were provided to each panelist per session. Between samples, panelists were instructed to take a bite of cracker and a sip of water in order to cleanse the palate. Additional crackers were provided if requested. The panel room was lit with red lights during each panel session. Once panelists finished with a coded sample, another coded sample followed approximately one minute later.

Panelists evaluated each milk sample for odor (overall intensity and off-odor intensity), flavor (overall intensity, off-flavor intensity, sweetness, aftertaste) and mouthfeel. Panelists recorded their assessment by making a mark on a line representing a continuum for that parameter. For example, the line for overall odor intensity went from bland to extremely intense. The values for the control reference were pre-marked on each line so that each parameter was assessed relative to the reference control. If an off-flavor was detected panelists could also record the type of off-flavor (flat, oxidized, acid/sour, rancid/bitter, malty, feed, fruity, salty, astringent, cooked, cowy/acetone, foreign) and the intensity (from one to four) for that particular off-flavor, one being slight, four being extreme. The written testing instructions and evaluation forms used for trained sensory evaluation are in Appendix 1.

Milk for consumer acceptability testing was taken from CTL, SAFF/M, and SAFF/ME on week nine. The milk was collected, processed, and stored in the same manner as that for the trained sensory evaluation. Potential panelists for untrained sensory evaluation were pre-screened based on the type of milk ordinarily consumed. Only individuals that typically consumed 2% or whole milk were selected for the panel. The evaluation was carried out on the day following processing and 76 panelists (55 females, 21 males; see Table 4.1 for demographic information) evaluated the milk for overall acceptability, flavour, and mouthfeel. The written testing instructions and evaluation forms used for the untrained sensory evaluation are in Appendix 2.

4.2.4 Statistical Analysis

Trained panel data was analyzed using the MIXED procedure of SAS version 8:3 (SAS Institute, Cary, NC). Treatment, week, storage time after processing, and all interactions were fixed effects. The Tukey's procedure was used to determine significance among means. Model effects were considered significant at $P<0.05$. The consumer panel data was analyzed using the General Linear Models procedure of SAS.

Treatment differences were evaluated using the Student-Newman-Keuls test with significance declared at $P<0.05$.

4.3 Results and Discussion

4.3.1 Fatty Acid Composition of Milk

The effect of dietary treatment on milk fatty acid composition was presented previously in Chapter 3. Milk from five cows on each treatment was taken and pooled for processing and trained sensory evaluation on weeks 0, 2, 4, and 8. The fatty acid composition of pooled milk from weeks 0, 2, 4, and 8 is presented in Tables 4.2 to 4.5 respectively. The profile of fatty acids in the pooled milk (which represented a subset of cows from each treatment) very closely mirrored the average composition for the whole group (Chapter 3 - Table 3.6; Figures 3.11-3.16), for each respective treatment at each time point.

Processing had no noticeable effect on milk fatty acid composition (Table 4.6). This is in contrast to a study by Campbell et al. (2003) who found that High-Temperature Short-Time (HTST) pasteurization (16 s at 77.2 °C) significantly reduced that concentration of cis-9, trans-11 CLA in CLA-enriched milk. This difference could be attributed to the lower pasteurization temperature (68 °C for 2.5 min) used in the study presented here. Another important difference was that the Campbell et al. (2003) study achieved CLA enrichment through fortification with synthetic CLA oil prior to homogenization. It is possible that this unesterified CLA was more susceptible to heat destruction than the natural CLA contained in the triglycerides and phospholipids of the milk fat globules.

4.3.2 Trained Panel Sensory Evaluation

A trained sensory panel evaluated milk from two days of each of weeks 0, 2, 4, and 8. The milk from each of these eight days underwent sensory evaluation the day after processing, and after five days storage at refrigeration temperatures. The results for this sensory evaluation are presented in Tables 4.7a to 4.20f.

At no time in the study did the identified reference control (REF) have a score that was different from the "hidden" experimental control (CTL). This provided assurance that the basic ingredients on which all the experimental diets were based were not causing any significant changes in the organoleptic characteristics of the milk, compared to milk from outside the experiment. This was important since the CTL diet was considered "typical" of a dairy diet fed in Alberta and the other treatments (containing supplemental oils, monensin and/or vitamin E) were being compared against the CTL milk.

There was no significant difference between any of the treatments (independent of week or storage time) for any of the wide range of sensory parameters measured (Part d, Tables 4.7 - 4.20). This demonstrates that the changes brought about by the experimental diets on fatty acid composition did not have a detectable effect on the organoleptic quality of the milk. This is in agreement with studies from the Minesota-South Dakota Dairy Foods Research Center (Baer et al., 2001; Ramaswamy et al., 2001). In these studies four experienced panelists evaluated the flavor of CLA-enriched milk (2.17 to 2.43 g CLA/100g fat) produced through fish oil and/or soybean feeding, compared to control milk (0.56 to 0.66 g CLA/100g fat). These studies concluded that there was no difference in flavor between the CLA-enriched milk and control. However, they appeared to be looking only for signs of oxidation. In contrast to studies that evaluated milk enriched naturally in CLA, Campbell et al. (2003) carried out trained sensory evaluation on milk fortified with a synthetic mixture of CLA isomers. Nine panelists evaluated CLA-fortified milk and scored the milk based on four sensory characteristics, which they named “milkfat”, “grassy/vegetable oil”, “sweet taste”, and “astringent” (Campbell et al., 2003). They found that the CLA-fortified milk had a significantly higher “grassy/vegetable oil” flavor and lower “milkfat” flavor compared to the 2% milk control. Since the researchers found no indicators of oxidation they attributed the particular flavor of CLA-fortified milk to the added CLA oil.

Although the overall treatment effect was not significant in this study, a significant week effect was observed for overall odor intensity (Table 4.7e), off odor intensity (Table 4.8e), overall flavor intensity (Table 4.9e), off flavor intensity (Table 4.10e), sweet intensity (Table 4.11e), aftertaste intensity (Table 4.12e), mouthfeel intensity (Table 4.13e), flat intensity (Table 4.14e), oxidized intensity (4.15e), rancid/bitter intensity (Table 4.17e), and feed intensity (Table 4.19e). This significance is the result of slightly higher scores obtained mostly in week four results and quite often from the SAFF and SAFF/ME treatments. The reason for this is not clear. However, although the significance suggests that these milk samples might be less acceptable, the differences are actually likely of little practical importance since they were small and mostly specific to week four.

Storing the milk for five days generally had no effect on the organoleptic characteristics of the milk compared to milk tested the day after processing (Part f, Tables 4.7 – 4.19). The exception to this was salty intensity, which showed a small but significantly higher score for day-5 verses day-0 at week 0 (Table 4.20f). However, this takes into account all treatments and is likely of no practical significance since individual treatments were not different between day-0 and day-5 on any week (Table 4.20a, 4.20b).

An important reason for doing the five day shelf-life evaluation was to test for off flavors or odors associated with oxidation since this is the problem most likely to occur in milk with elevated levels of polyunsaturated fatty acids (PUFA) (Ashes et al., 1997). A good example that highlights this problem was a study by Goering et al. (1976) who showed that feeding protected safflower oil increased the 18:2 n-6 in milk fat and resulted in the development of strong oxidized flavors. Many studies have demonstrated that enhancing the concentration of vitamin E in milk through dietary supplementation of vitamin E (most often α-tocopherol) prevents oxidation in milk with elevated PUFA (Atwal et al., 1991; Nicholson and St-Laurent, 1991; Ashes et al., 1997; Focant et al., 1998). Feeding oilseeds without supplemental vitamin E also increases the vitamin E content of bovine milk to a small extent, most likely because of the natural tocopherols present in the oil (Focant et al., 1998). However, Focant et al. (1998) found that this increase in vitamin E was not sufficient to protect against oxidation in the milk of cows whose diets were supplemented with rapeseed or linseed. Furthermore, of particular relevance to the study presented in this chapter, Yang et al. (2000) found that CLA was more susceptible to autooxidation than linoleic acid (18:2 n-6), suggesting that the conjugated double bond was more susceptible to oxidation than the methylene interrupted double bond. Vitamin E was supplemented in a number of the diets used in the present study in anticipation that CLA-enriched milk would be more prone to oxidation than CTL milk. The author is not aware of any other study that has evaluated the oxidative stability of milk with CLA at the levels obtained in this study. Therefore, the degree of oxidation, if any, to expect after five days of storage was difficult to anticipate. As noted above, the study presented here did not find any off flavors or odors associated with oxidation after five days of storage, with or without elevated vitamin E. It is likely that, even after five days, the milk was simply too fresh to detect oxidation.

4.3.3 Untrained Consumer Evaluation

The fatty acid composition of the CTL, SAFF/M, and SAFF/ME milk used for the consumer evaluation is shown in Table 4.21. The values are similar to those reported at week 8 for the respective treatments (Chapter 3, Figures 3.11-3.16). The results of the consumer evaluation are shown in Table 4.22. Compared to CTL, SAFF/M milk scored significantly lower for overall acceptability ($P<0.05$) and flavor ($P<0.001$) whereas SAFF/ME milk was not different from CTL. Mouthfeel was not significantly different between any of the treatments. The reason for the difference between SAFF/M and SAFF/ME is not clear since the fatty acid composition was not particularly different between the two treatments (Table 4.21). The panelists had the opportunity to provide written comments on characteristics they "like" or "disliked" about each milk sample. These are presented in Tables 4.23a and 4.23b respectively. Overall, the number of positive comments outweighed the negative comments for each treatment. However, the comments give no clue as to why SAFF/M scored lower for acceptability and flavor, as there appeared to be no consistency in the comments. Despite this, the results did show that CLA-enriched milk (at least for SAFF/ME) had a similar overall acceptability, flavor, and mouthfeel as the CTL milk.

To date, there have been only two other studies looking at consumer evaluation of CLA-enriched milk (Ramaswamy et al., 2001; Campbell et al., 2003). The study by Ramaswamy et al. (2001) compared milk with 0.56 g CLA/100g fat with milk that contained 2.3 g CLA/100g fat, produced by including fish oil in the dairy diet. This study found no difference in acceptability or flavor between treatments. However, the panelists in this study did not directly compare the two milk samples but actually tasted one or the other and rated its acceptability on a scale from poor to excellent (Ramaswamy et al., 2001). In the study by Campbell et al. (2003) the consumers evaluated CLA-fortified milks (2%-CLA, 1%-CLA:1% milk fat) with milk not fortified with CLA (2% milk fat). Each panelist evaluated all three milk samples for overall acceptability, overall flavor, and freshness. Milk fortified with 2% CLA scored lower for overall acceptability, flavor, and perceived freshness compared to the 2% milk fat. The results for the consumer evaluation (as well as the trained sensory evaluation) suggest that fortifying milk with synthetic CLA results in a product with less acceptable organoleptic characteristics (Campbell et al., 2003).

4.4 Conclusion

This study evaluated the organoleptic characteristics and acceptability of fresh processed CLA-enriched milk using both trained sensory evaluation and untrained consumer testing. The trained sensory panel found that the milk samples with higher CLA were not different to CTL for any of the wide range of sensory parameters evaluated. This suggested that CLA-enriched milk would be an equally acceptable product for fluid milk consumption as the CTL, which represented "typical" Alberta milk.

The untrained panel found that the SAFF/M CLA-enriched milk sample was less acceptable than CTL, whereas the SAFF/ME CLA-enriched milk was as acceptable as CTL. The reason for this difference between SAFF/M and SAFF/ME was not clear although it was not likely due to differences in fatty acid composition since the fatty acid profiles of SAFF/M and SAFF/ME were quite similar.

Table 4.1 Demographic information of untrained sensory panelists

Age category	Male	Female	Total
18-24	6 (8%)	11 (14.5%)	17 (22%)
25-34	2 (3%)	11 (14.5%)	13 (17%)
35-44	6 (8%)	11 (14.5%)	17 (22%)
45-54	4 (5%)	17 (22%)	21 (28%)
55-64	0 (0%)	4 (5%)	4 (5%)
65+	3 (4%)	1 (1%)	4 (5%)
Total	21 (28%)	55 (72%)	76 (100%)

Table 4.2 Milk fatty acid composition of week 0 unprocessed pre-treatment pooled milk used for trained sensory evaluation

	Treatment[1,2]					
Fatty Acid[3]	CTL (CTL)	CTL (SAFF)	CTL (SAFF/E)	CTL (SAFF/M)	CTL (SAFF/ME)	CTL (FLAX/E)
4:0	3.88	4.20	4.35	4.60	4.59	3.99
6:0	2.26	2.40	2.25	2.43	2.22	2.21
8:0	1.17	1.26	1.16	1.29	1.14	1.15
10:0	2.60	2.74	2.51	2.89	2.55	2.55
11:0	0.33	0.29	0.29	0.34	0.29	0.32
12:0	2.98	3.02	2.82	3.26	2.89	2.93
13:0	0.17	0.17	0.16	0.20	0.19	0.19
14:0	10.53	10.58	9.95	11.25	10.31	10.89
14:1	0.80	0.64	0.68	0.72	0.61	0.79
15:0	1.11	1.05	1.10	1.22	1.15	1.16
16:0	31.58	28.50	28.21	29.61	28.45	30.24
16:1	1.53	1.34	1.56	1.42	1.47	1.33
18:0	10.51	12.07	12.26	11.40	12.20	11.15
18:1 trans	2.38	2.52	2.39	1.94	1.93	2.35
18:1 n-9	19.79	20.66	21.85	19.78	22.22	20.26
18:1 n-7	0.49	0.58	0.60	0.59	0.59	0.54
18:2 t-11, c-15	0.38	0.40	0.40	0.36	0.34	0.42
18:2 n-6	1.77	1.86	1.91	1.67	1.81	1.77
18:3 n-3	0.43	0.42	0.40	0.39	0.37	0.40
20:0	0.20	0.23	0.22	0.21	0.23	0.22
20:1 n-12	0.16	0.16	0.16	0.16	0.16	0.17
20:1 n-9	0.08	0.09	0.07	0.06	0.07	0.08
CLA c-9, t-11	0.57	0.53	0.48	0.40	0.41	0.54
CLA t-10, c-12	nd	nd	nd	nd	nd	nd
Other FA	4.32	4.30	4.22	3.81	3.79	4.36

[1] All cows were fed the control diet (CTL) during week 0; Name in parenthesis indicates the treatment each group subsequently received: control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E).

[2] Values for each treatment represent pooled milk from five cows

[3] Fatty acids are expressed as a percentage of fatty acid methyl esters

Table 4.3 Milk fatty acid composition of week 2 unprocessed treatment-pooled milk used for trained sensory evaluation

	Treatment[1, 2]					
Fatty Acid[3]	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E
4:0	4.24	3.19	3.71	3.36	3.40	3.43
6:0	2.51	1.64	1.79	1.84	1.64	1.75
8:0	1.31	0.75	0.79	0.80	0.74	0.80
10:0	2.92	1.49	1.52	1.52	1.46	1.53
11:0	0.36	0.15	0.18	0.18	0.16	0.22
12:0	3.26	1.69	1.75	1.75	1.69	1.79
13:0	0.22	0.12	0.13	0.11	0.11	0.14
14:0	11.94	8.07	8.48	8.52	8.66	8.76
14:1	0.90	0.46	0.57	0.54	0.48	0.63
15:0	1.09	0.70	0.76	0.81	0.76	0.79
16:0	32.27	17.58	17.46	17.60	17.77	17.44
16:1	1.61	0.83	0.95	0.85	0.90	0.89
18:0	9.37	12.08	12.70	10.25	10.46	10.86
18:1 trans	2.63	14.94	10.95	16.49	15.88	9.35
18:1 n-9	17.03	20.08	23.21	18.56	19.73	22.93
18:1 n-7	0.47	0.59	0.54	0.61	0.55	0.56
18:2 t-11, c-15	0.41	0.55	0.52	0.62	0.54	3.06
18:2 n-6	1.60	2.97	2.64	2.82	2.78	1.97
18:3 n-3	0.39	0.35	0.38	0.37	0.34	0.73
20:0	0.18	0.17	0.18	0.16	0.17	0.52
20:1 n-12	0.17	0.12	0.12	0.14	0.12	0.16
20:1 n-9	0.08	0.08	0.07	0.10	0.08	0.22
CLA c-9, t-11	0.63	4.06	2.88	4.36	4.68	2.81
CLA t-10, c-12	nd	0.03	0.05	0.07	0.04	nd
Other FA	4.42	7.31	7.69	7.54	6.87	8.67

[1] Diets were control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E).

[2] Values for each treatment represent pooled milk from five cows

[3] Fatty acids are expressed as a percentage of fatty acid methyl esters

Table 4.4 Milk fatty acid composition of week 4 unprocessed treatment-pooled milk used for trained sensory evaluation

	Treatment[1,2]					
Fatty Acid[3]	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E
4:0	3.77	2.85	2.71	2.84	2.78	3.21
6:0	2.36	1.26	1.31	1.43	1.38	1.52
8:0	1.19	0.55	0.61	0.63	0.63	0.67
10:0	2.61	1.07	1.19	1.23	1.25	1.33
11:0	0.34	0.12	0.15	0.13	0.17	0.18
12:0	2.98	1.35	1.50	1.50	1.51	1.55
13:0	0.20	0.08	0.11	0.12	0.13	0.11
14:0	11.34	6.54	7.11	7.71	7.49	7.48
14:1	0.82	0.45	0.58	0.51	0.53	0.54
15:0	1.11	0.73	0.74	0.82	0.78	0.78
16:0	31.92	17.71	16.96	18.30	18.93	17.48
16:1	1.50	1.00	0.96	1.05	1.07	0.95
18:0	10.19	13.06	11.98	10.46	10.48	12.44
18:1 trans	3.06	14.66	15.73	17.69	17.41	8.95
18:1 n-9	17.87	22.72	22.22	19.41	19.83	24.61
18:1 n-7	0.46	0.54	0.52	0.63	0.53	0.52
18:2 t-11, c-15	0.45	0.52	0.52	0.55	0.50	3.12
18:2 n-6	1.71	2.95	2.93	2.90	2.80	2.05
18:3 n-3	0.38	0.29	0.33	0.32	0.27	0.74
20:0	0.20	0.19	0.17	0.18	0.16	0.50
20:1 n-12	0.15	0.13	0.13	0.11	0.11	0.17
20:1 n-9	0.06	0.08	0.09	0.12	0.10	0.22
CLA c-9, t-11	0.72	4.07	3.97	4.54	4.89	2.62
CLA t-10, c-12	nd	0.02	0.06	0.08	0.09	nd
Other FA	4.60	7.05	7.38	6.76	6.16	8.26

[1] Diets were control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E).

[2] Values for each treatment represent pooled milk from five cows

[3] Fatty acids are expressed as a percentage of fatty acid methyl esters

Table 4.5 Milk fatty acid composition of week 8 unprocessed treatment-pooled milk used for trained sensory evaluation

	Treatment[1,2]					
Fatty Acid[3]	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E
4:0	4.37	2.83	2.67	2.91	2.68	3.27
6:0	2.47	1.29	1.27	1.34	1.36	1.53
8:0	1.26	0.56	0.56	0.58	0.62	0.67
10:0	2.71	1.07	1.09	1.15	1.24	1.27
11:0	0.35	0.10	0.13	0.12	0.13	0.17
12:0	3.07	1.32	1.42	1.46	1.50	1.49
13:0	0.20	0.09	0.13	0.10	0.10	0.11
14:0	12.41	8.09	8.31	9.14	8.27	8.58
14:1	0.98	0.49	0.70	0.58	0.50	0.60
15:0	1.07	0.69	0.73	0.76	0.78	0.73
16:0	32.18	17.39	17.02	17.71	18.02	17.09
16:1	1.56	0.77	0.97	0.83	0.82	0.75
18:0	9.08	12.82	12.00	9.60	11.19	10.93
18:1 trans	2.35	14.84	13.88	19.86	15.76	8.60
18:1 n-9	17.56	21.80	23.35	17.59	20.67	24.28
18:1 n-7	0.42	0.49	0.57	0.65	0.53	0.51
18:2 t-11, c-15	0.40	0.50	0.51	0.57	0.51	3.70
18:2 n-6	1.66	2.81	3.09	2.99	3.03	2.12
18:3 n-3	0.38	0.29	0.31	0.31	0.32	0.82
20:0	0.20	0.19	0.18	0.17	0.18	0.65
20:1 n-12	0.17	0.13	0.14	0.12	0.14	0.18
20:1 n-9	0.07	0.09	0.08	0.12	0.09	0.31
CLA c-9, t-11	0.68	4.26	3.35	4.71	4.87	2.82
CLA t-10, c-12	0.01	0.04	0.10	0.10	0.05	0.01
Other FA	4.39	7.03	5.97	6.55	6.63	8.80

[1] Diets were control (CTL), and, control supplemented with: safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), flaxseed plus vitamin E (FLAX/E)

[2] Values for each treatment represent pooled milk from five cows

[3] Fatty acids are expressed as a percentage of fatty acid methyl esters

Table 4.6 - Milk fatty acid composition of unprocessed verses processed milk.

	Milk		
Fatty Acid[1,2]	UN-PROCESSED	PROCESSED	% DIFFERENCE
4:0	2.95	3.01	+ 2.11
6:0	1.65	1.65	+ 0.43
8:0	0.79	0.79	+ 0.93
10:0	1.63	1.65	+ 0.97
11:0	0.17	0.17	- 0.04
12:0	1.93	1.95	+ 0.97
13:0	0.11	0.11	+ 2.79
14:0	7.51	7.59	+ 1.10
14:1	0.63	0.63	+ 0.01
15:0	0.80	0.80	+ 0.31
16:0	20.50	20.54	+ 0.15
16:1	0.91	0.90	- 0.54
18:0	10.52	10.51	- 0.05
18:1 trans	15.06	15.37	+ 2.07
18:1 n-9	18.96	18.74	- 1.20
18:1 n-7	0.55	0.54	- 1.75
18:2 t-11, c-15	0.57	0.55	- 2.89
18:2 n-6	2.66	2.65	- 0.68
18:3 n-3	0.34	0.34	- 2.40
20:0	0.17	0.17	- 2.65
20:1 n-12	0.14	0.13	- 3.74
20:1 n-9	0.10	0.10	- 2.51
CLA c-9, t-11	4.02	4.05	+ 0.78
CLA t-10, c-12	0.05	0.05	- 5.12
Other FA	7.28	7.00	- 3.85

[1] Fatty acid analysis was carried out on unprocessed and processed SAFF/M (week 2), SAFF/ME (week 2), SAFF/E (week4), CTL (week9), SAFF/M (week 9) and SAFF/ME (week 9) treatment-pooled milk samples. Each reported fatty acid value represents the average value for these samples and not the fatty acid composition of any particular treatment.

[2] Fatty acids are expressed as a percentage of fatty acid methyl esters

Table 4.7a - Overall Odor Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	4.8	4.6	4.7	4.8	4.6	4.7	4.9
	2	4.5	4.7	4.4	4.7	4.9	5.0	4.9
	4	5.0	5.5	4.9	4.9	6.0	4.8	4.4
	8	4.5	4.9	4.4	4.8	4.6	4.7	4.5
5 days	0	4.7	4.9	4.5	5.1	4.8	4.4	4.8
	2	4.5	4.5	5.0	5.1	4.9	4.6	4.6
	4	4.8	5.7	4.7	5.4	5.8	4.5	4.5
	8	4.5	4.5	4.4	4.6	4.5	4.4	4.4

[1] Standard Error of the Mean = 0.28, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.7b - Overall Odor Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	4.7	4.9	4.6	4.8	5.0	4.8	4.7
5 days	4.6	4.9	4.7	5.1	5.0	4.5	4.6

[1] Standard Error of the Mean = 0.18, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.7c - Overall Odor Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	4.7^{a}	4.7^{a}	4.6^{a}	5.0^{a}	4.7^{a}	4.6^{a}	4.9^{a}
2	4.5^{a}	4.6^{a}	4.7^{a}	4.9^{a}	4.9^{a}	4.8^{a}	4.7^{a}
4	4.9^{abc}	5.6^{ab}	4.8^{abc}	5.1^{abc}	5.9^{a}	4.7^{bc}	4.5^{c}
8	4.5^{a}	4.7^{a}	4.4^{a}	4.7^{a}	4.6^{a}	4.6^{a}	4.5^{a}

[1] Standard Error of the Mean= 0.22, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

[abc] Means within the same row sharing a common letter are not significantly different from each other , $p<0.05$

Table 4.7d - Overall Odor Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
4.6	4.9	4.6	4.9	5.0	4.7	4.6

[1] Standard Error of the Mean = 0.16, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.7e - Overall Odor Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
4.7^{b}	4.7^{b}	5.1^{a}	4.6^{b}

[1] Standard Error of the Mean = 0.08, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.7f - Overall Odor Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	4.7
0	2	4.7
0	4	5.1
0	8	4.6
5	0	4.7
5	2	4.7
5	4	5.1
5	8	4.5

[1] Standard Error of the Mean = 0.11, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.8a - Off Odor Intensity: Treatment means after 0 or 5 days storage for each week.

Storage Time	Week	Treatment Means[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	1.1	1.0	1.5	1.2	1.2	1.1	1.5
	2	1.2	1.1	1.0	1.3	1.2	1.3	1.5
	4	1.1	2.9	1.4	1.2	3.0	1.5	0.9
	8	1.0	1.3	0.9	1.5	1.0	1.1	1.0
5 days	0	1.1	1.2	1.0	1.3	1.2	0.9	1.2
	2	0.9	0.9	1.2	1.7	1.3	1.5	1.1
	4	1.3	2.7	1.1	2.1	2.9	1.2	1.0
	8	0.9	1.0	0.9	1.2	1.1	0.8	1.0

[1] Standard Error of the Mean = 0.37, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.8b - Off Odor Intensity: Treatment means after 0 or 5 days storage independent of week.

Storage Time	Treatment Means by Storage Time[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	1.1	1.6	1.2	1.3	1.6	1.3	1.2
5 days	1.0	1.5	1.1	1.6	1.6	1.1	1.1

[1] Standard Error of the Mean = 0.26, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.8c - Off Odor Intensity: Treatment means for each week independent of storage time.

Week	Treatment Means by Week[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	1.1^{a}	1.1^{a}	1.2^{a}	1.2^{a}	1.2^{a}	1.0^{a}	1.3^{a}
2	1.0^{a}	1.0^{a}	1.1^{a}	1.5^{a}	1.2^{a}	1.4^{a}	1.3^{a}
4	1.2^{b}	2.8^{a}	1.3^{b}	1.6^{ab}	2.9^{a}	1.4^{b}	1.0^{b}
8	0.9^{a}	1.2^{a}	0.9^{a}	1.4^{a}	1.1^{a}	1.0^{a}	1.0^{a}

[1] Standard Error of the Mean = 0.30, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.8d - Off Odor Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
1.1	1.5	1.1	1.4	1.6	1.2	1.1

[1] Standard Error of the Mean = 0.24, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.8e - Off Odor Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
1.2^b	1.2^b	1.7^a	1.0^b

[1] Standard Error of the Mean = 0.11, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.8f - Off Odor Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	1.2
0	2	1.2
0	4	1.7
0	8	1.1
5	0	1.1
5	2	1.3
5	4	1.7
5	8	1.0

[1] Standard Error of the Mean = 0.14, not significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.9a - Overall Flavor Intensity: Treatment means after 0 or 5 days storage for each week.

Storage Time	Week	Treatment Means[1,2]						
		CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	7.8	7.5	7.7	7.9	7.4	8.5	7.9
	2	7.6	7.5	7.4	7.7	8.0	7.9	7.9
	4	7.9	8.6	8.3	8.4	8.7	8.0	7.6
	8	7.8	8.0	7.6	8.1	7.9	8.2	7.7
5 days	0	8.2	8.1	7.7	8.1	8.0	7.7	7.5
	2	7.4	7.3	7.7	8.1	7.6	7.7	7.7
	4	7.6	8.6	7.9	8.7	8.4	8.1	7.5
	8	7.5	7.6	7.6	8.0	7.8	7.8	7.8

[1] Standard Error of the Mean = 0.35, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.9b - Overall Flavor Intensity: Treatment means after 0 or 5 days storage independent of week.

Storage Time	Treatment Means by Storage Time[1,2]						
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	7.8	7.9	7.7	8.0	8.0	8.2	7.8
5 days	7.7	7.9	7.7	8.2	8.0	7.8	7.6

[1] Standard Error of the Mean = 0.22, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.9c - Overall Flavor Intensity: Treatment means for each week independent of storage time.

Week	Treatment Means by Week[1,2]						
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	8.0	7.8	7.7	8.0	7.7	8.1	7.7
2	7.5	7.4	7.6	7.9	7.8	7.8	7.8
4	7.8	8.6	8.1	8.5	8.6	8.1	7.6
8	7.7	7.8	7.6	8.0	7.9	8.0	7.8

[1] Standard Error of the Mean = 0.27, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.9d - Overall Flavor Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
7.7	7.9	7.7	8.1	8.0	8.0	7.7

[1]Standard Error of the Mean = 0.19, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2]Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.9e - Overall Flavor Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
7.9^b	7.7^b	8.2^a	7.8^b

[1]Standard Error of the Mean = 0.10, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.9f - Overall Flavor Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	7.8
0	2	7.7
0	4	8.2
0	8	7.9
5	0	7.9
5	2	7.6
5	4	8.1
5	8	7.7

[1]Standard Error of the Mean = 0.13, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.10a – Off Flavor Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	1.8	1.8	2.6	1.4	1.7	2.8	2.5
	2	1.6	1.5	1.7	2.0	2.3	1.7	1.9
	4	2.0	3.2	2.1	2.4	3.1	2.3	1.5
	8	1.7	2.0	1.2	2.0	1.4	2.1	1.6
5 days	0	2.1	1.9	1.6	1.9	2.2	1.6	1.6
	2	1.4	1.7	2.2	2.2	1.8	2.3	1.8
	4	1.5	3.2	1.5	2.6	2.8	1.7	1.5
	8	1.3	1.3	1.2	1.8	1.6	1.7	1.8

[1] Standard Error of the Mean = 0.49, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.10b – Off Flavor Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	1.8	2.1	1.9	2.0	2.1	2.2	1.9
5 days	1.6	2.0	1.6	2.2	2.1	1.8	1.7

[1] Standard Error of the Mean = 0.28, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.10c – Off Flavor Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	2.0^{a}	1.8^{a}	2.1^{a}	1.7^{a}	1.9^{a}	2.2^{a}	2.1^{a}
2	1.5^{a}	1.6^{a}	2.0^{a}	2.1^{a}	2.1^{a}	2.0^{a}	1.8^{a}
4	1.8^{a}	3.2^{a}	1.8^{a}	2.5^{a}	3.0^{a}	2.0^{a}	1.5^{a}
8	1.5^{a}	1.7^{a}	1.2^{a}	1.9^{a}	1.5^{a}	1.9^{a}	1.7^{a}

[1] Standard Error of the Mean = 0.036, significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.10d – Off Flavor Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
1.7	2.1	1.8	2.1	2.1	2.0	1.8

[1] Standard Error of the Mean = 0.24, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.10e – Off Flavor Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
2.0^{ab}	1.9^{ab}	2.3^{a}	1.6^{b}

[1] Standard Error of the Mean = 0.14, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.10f – Off Flavor Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	2.1
0	2	1.8
0	4	2.4
0	8	1.7
5	0	1.8
5	2	1.9
5	4	2.1
5	8	1.5

[1] Standard Error of the Mean = 0.18, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.11a - Sweet Intensity: Treatment means after 0 or 5 days storage for each week.

Storage Time	Week	Treatment Means[1,2]						
		CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	4.1	3.9	3.4	4.3	3.7	4.1	3.6
	2	3.8	3.7	3.7	3.8	3.6	3.6	4.2
	4	3.9	3.8	3.9	4.1	3.6	4.1	4.0
	8	4.1	3.5	4.2	3.6	4.2	4.1	4.1
5 days	0	4.5	4.0	4.0	4.1	3.6	3.9	4.1
	2	3.7	3.3	3.2	3.3	3.7	3.8	3.6
	4	4.0	3.0	4.1	3.2	3.6	4.0	3.8
	8	3.9	3.8	4.1	3.8	3.9	4.1	3.9

[1] Standard Error of the Mean = 0.26, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.11b - Sweet Intensity: Treatment means after 0 or 5 days storage independent of week.

Storage Time	Treatment Means by Storage Time[1,2]						
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	4.0	3.7	3.8	3.9	3.8	4.0	4.0
5 days	4.1	3.5	3.8	3.6	3.7	4.0	3.8

[1] Standard Error of the Mean = 0.12, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.11c - Sweet Intensity: Treatment means for each week independent of storage time.

Week	Treatment Means by Week[1,2]						
	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	4.3	4.0	3.7	4.2	3.7	4.0	3.9
2	3.8	3.5	3.5	3.5	3.6	3.7	3.9
4	4.0	3.4	4.0	3.6	3.6	4.0	3.9
8	4.0	3.6	4.2	3.7	4.1	4.1	4.0

[1] Standard Error of the Mean = 0.18, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.11d - Sweet Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
4.0	3.6	3.8	3.8	3.7	4.0	3.9

[1] Standard Error of the Mean = 0.09, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.11e - Sweet Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
4.0^a	3.6^b	3.8^{ab}	4.0^a

[1] Standard Error of the Mean = 0.07, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other , $p<0.05$

Table 4.11f - Sweet Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	3.9
0	2	3.8
0	4	3.9
0	8	4.0
5	0	4.0
5	2	3.5
5	4	3.6
5	8	3.9

[1] Standard Error of the Mean = 0.10, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.12a - Aftertaste Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	4.6	4.3	4.2	4.3	3.9	5.0	4.9
	2	4.2	3.9	4.0	4.1	4.2	4.3	4.5
	4	4.2	5.9	4.7	5.0	5.5	4.8	4.1
	8	4.4	4.6	4.1	4.7	4.3	4.6	4.5
5 days	0	4.3	4.6	4.3	4.4	4.9	4.4	3.7
	2	3.9	4.2	4.5	5.3	4.4	4.7	4.4
	4	4.1	5.5	4.5	5.5	5.0	4.6	3.9
	8	4.4	4.2	4.3	4.7	4.1	4.4	4.5

[1] Standard Error of the Mean = 0.43, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.12b - Aftertaste Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	4.4	4.7	4.2	4.5	4.5	4.7	4.5
5 days	4.2	4.6	4.4	5.0	4.6	4.5	4.1

[1] Standard Error of the Mean = 0.26, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.12c - Aftertaste Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	4.4^{a}	4.5^{a}	4.3^{a}	4.4^{a}	4.4^{a}	4.7^{a}	4.3^{a}
2	4.1^{a}	4.0^{a}	4.2^{a}	4.7^{a}	4.3^{a}	4.5^{a}	4.5^{a}
4	4.2^{ab}	5.7^{a}	4.6^{ab}	5.2^{ab}	5.2^{ab}	4.7^{ab}	4.0^{b}
8	4.4^{a}	4.4^{a}	4.2^{a}	4.7^{a}	4.2^{a}	4.5^{a}	4.5^{a}

[1] Standard Error of the Mean = 0.33, significant, $p<0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.12d - Aftertaste Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
4.3	4.6	4.3	4.8	4.5	4.6	4.3

[1] Standard Error of the Mean = 0.23, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.12e - Aftertaste Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
4.4^b	4.3^b	4.8^a	4.4^b

[1] Standard Error of the Mean = 0.12, significant, $p<0.01$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.12f - Aftertaste Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	4.5
0	2	4.2
0	4	4.9
0	8	4.5
5	0	4.4
5	2	4.5
5	4	4.7
5	8	4.4

[1] Standard Error of the Mean = 0.16, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.13a – Mouth Feel Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	5.8	5.3	5.4	5.9	5.1	5.7	5.4
	2	5.4	5.2	5.3	5.2	5.5	5.6	5.5
	4	5.4	6.0	5.8	5.9	5.8	5.6	5.3
	8	5.4	5.7	5.6	5.8	5.6	5.4	5.3
5 days	0	5.7	5.5	5.5	5.5	5.6	5.5	5.2
	2	5.3	5.0	5.6	5.5	5.6	5.2	5.3
	4	5.7	5.7	5.7	5.9	5.7	5.6	5.2
	8	5.6	5.5	5.7	5.8	5.5	5.4	5.7

[1] Standard Error of the Mean = 0.22, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.13b – Mouth Feel Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	5.5	5.6	5.5	5.7	5.2	5.6	5.4
5 days	5.6	5.5	5.6	5.7	5.6	5.4	5.4

[1] Standard Error of the Mean = 0.11, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.13c – Mouth Feel Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	5.7	5.4	5.4	5.7	5.3	5.6	5.3
2	5.3	5.1	5.4	5.4	5.6	5.4	5.4
4	5.5	5.8	5.7	5.9	5.8	5.6	5.3
8	5.5	5.6	5.6	5.8	5.5	5.4	5.5

[1] Standard Error of the Mean = 0.16, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.13d – Mouth Feel Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
5.5	5.5	5.6	5.7	5.6	5.5	5.4

[1] Standard Error of the Mean = 0.09, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.13e – Mouth Feel Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
5.5^{ab}	5.4^{b}	5.7^{a}	5.6^{a}

[1] Standard Error of the Mean = 0.06, significant, $p<0.001$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.13f – Mouth Feel Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	5.5
0	2	5.4
0	4	5.7
0	8	5.6
5	0	5.5
5	2	5.3
5	4	5.6
5	8	5.6

[1] Standard Error of the Mean = 0.08, not significant, $p>0.05$; Score is in cm on a scale of 0 to 15 cm, see Appendix 1

Table 4.14a - Flat Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.2	0.1	0.2	0.0	0.3	0.0	0.0
	2	0.1	0.2	0.3	0.1	0.2	0.1	0.1
	4	0.1	0.1	0.0	0.0	0.1	0.1	0.1
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.1
5 days	0	0.1	0.2	0.0	0.0	0.2	0.1	0.2
	2	0.1	0.2	0.2	0.0	0.1	0.2	0.1
	4	0.0	0.1	0.0	0.0	0.0	0.0	0.2
	8	0.2	0.1	0.0	0.1	0.2	0.1	0.1

[1] Standard Error of the Mean = 0.09, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.14b - Flat Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.1	0.1	0.1	0.0	0.1	0.0	0.1
5 days	0.1	0.1	0.1	0.0	0.1	0.1	0.2

[1] Standard Error of the Mean = 0.05, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.14c - Flat Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.1	0.2	0.1	0.0	0.2	0.1	0.1
2	0.1	0.2	0.3	0.0	0.2	0.1	0.1
4	0.0	0.1	0.0	0.0	0.0	0.0	0.2
8	0.1	0.0	0.0	0.0	0.1	0.0	0.1

[1] Standard Error of the Mean = 0.06, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.14d - Flat Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.1	0.1	0.1	0.0	0.1	0.1	0.1

[1] Standard Error of the Mean = 0.03, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.14e - Flat Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.12^{ab}	0.14^{a}	0.05^{b}	0.06^{b}

[1] Standard Error of the Mean = 0.03, significant, $p<0.001$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.14f - Flat Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.1
0	2	0.2
0	4	0.1
0	8	0.0
5	0	0.1
5	2	0.1
5	4	0.0
5	8	0.1

[1] Standard Error of the Mean = 0.04, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.15a - Oxidized Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.0	0.0	0.1	0.3	0.0	0.0	0.4
	2	0.1	0.1	0.4	0.1	0.4	0.3	0.3
	4	0.3	0.0	0.3	0.5	0.3	0.3	0.2
	8	0.0	0.3	0.0	0.3	0.3	0.2	0.1
5 days	0	0.0	0.1	0.1	0.1	0.4	0.0	0.0
	2	0.2	0.1	0.5	0.5	0.0	0.5	0.2
	4	0.2	0.3	0.0	0.6	0.1	0.3	0.1
	8	0.1	0.2	0.0	0.3	0.1	0.1	0.3

[1] Standard Error of the Mean = 0.15, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.15b - Oxidized Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.1	0.1	0.2	0.3	0.2	0.2	0.2
5 days	0.1	0.2	0.1	0.4	0.1	0.2	0.1

[1] Standard Error of the Mean = 0.08, not significant, p>0.05 ; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.15c - Oxidized Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.0	0.1	0.1	0.2	0.2	0.0	0.2
2	0.2	0.1	0.4	0.3	0.2	0.4	0.2
4	0.2	0.2	0.1	0.5	0.2	0.3	0.1
8	0.0	0.3	0.0	0.3	0.2	0.1	0.2

[1] Standard Error of the Mean = 0.11, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.15d - Oxidized Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.1	0.1	0.2	0.3	0.2	0.2	0.2

[1] Standard Error of the Mean = 0.06, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.15e - Oxidized Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.10^b	0.25^a	0.24^{ab}	0.15^{ab}

[1] Standard Error of the Mean = 0.04, significant, $p<0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.15f - Oxidized Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.1
0	2	0.2
0	4	0.3
0	8	0.1
5	0	0.1
5	2	0.3
5	4	0.2
5	8	0.2

[1] Standard Error of the Mean = 0.06, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.16a – Acid Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.0	0.2	0.0	0.0	0.1	0.4	0.0
	2	0.2	0.0	0.0	0.2	0.0	0.2	0.2
	4	0.2	0.5	0.1	0.0	0.2	0.1	0.0
	8	0.0	0.0	0.0	0.1	0.0	0.2	0.3
5 days	0	0.1	0.1	0.0	0.1	0.0	0.1	0.0
	2	0.0	0.1	0.0	0.0	0.0	0.0	0.2
	4	0.0	0.4	0.0	0.1	0.1	0.0	0.1
	8	0.1	0.0	0.0	0.0	0.0	0.3	0.0

[1] Standard Error of the Mean = 0.13, not significant, $p > 0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.16b – Acid Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.1	0.2	0.0	0.1	0.1	0.2	0.1
5 days	0.1	0.2	0.0	0.1	0.0	0.1	0.1

[1] Standard Error of the Mean = 0.06, not significant, $p > 0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.16c – Acid Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.1^{a}	0.1^{a}	0.0^{a}	0.1^{a}	0.0^{a}	0.3^{a}	0.0^{a}
2	0.1^{a}	0.0^{a}	0.0^{a}	0.1^{a}	0.0^{a}	0.1^{a}	0.2^{a}
4	0.1^{a}	0.5^{a}	0.1^{a}	0.1^{a}	0.2^{a}	0.0^{a}	0.1^{a}
8	0.1^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.3^{a}	0.1^{a}

[1] Standard Error of the Mean = 0.10, significant, $p < 0.01$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p < 0.05$

Table 4.16d – Acid Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.1	0.2	0.0	0.1	0.1	0.2	0.1

[1] Standard Error of the Mean = 0.07, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.16e – Acid Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.1	0.1	0.1	0.1

[1] Standard Error of the Mean = 0.04, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.16f – Acid Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.1
0	2	0.1
0	4	0.2
0	8	0.1
5	0	0.1
5	2	0.0
5	4	0.1
5	8	0.1

[1] Standard Error of the Mean = 0.05, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.17a – Rancid/Bitter Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.3	0.2	0.3	0.0	0.2	0.3	0.3
	2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	4	0.1	0.1	0.0	0.0	0.1	0.1	0.2
	8	0.3	0.3	0.0	0.1	0.0	0.0	0.0
5 days	0	0.1	0.1	0.2	0.0	0.3	0.1	0.0
	2	0.0	0.2	0.0	0.1	0.2	0.2	0.1
	4	0.1	0.0	0.1	0.1	0.0	0.1	0.0
	8	0.0	0.1	0.0	0.0	0.1	0.0	0.0

[1] Standard Error of the Mean = 0.09, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.17b – Rancid/Bitter Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.2	0.2	0.1	0.0	0.1	0.1	0.1
5 days	0.1	0.1	0.1	0.0	0.1	0.1	0.0

[1] Standard Error of the Mean = 0.04 not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.17c – Rancid/Bitter Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.2	0.1	0.2	0.0	0.3	0.2	0.1
2	0.0	0.1	0.0	0.1	0.1	0.1	0.1
4	0.1	0.1	0.0	0.0	0.0	0.1	0.1
8	0.1	0.2	0.0	0.1	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.07, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.17d – Rancid/Bitter Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.1	0.1	0.1	0.0	0.1	0.1	0.1

[1] Standard Error of the Mean = 0.03, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.17e – Rancid/Bitter Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.20^a	0.08^{ab}	0.07^b	0.06^b

[1] Standard Error of the Mean = 0.02, significant, $p<0.01$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.17f – Rancid/Bitter Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.2
0	2	0.1
0	4	0.1
0	8	0.1
5	0	0.1
5	2	0.1
5	4	0.1
5	8	0.0

[1] Standard Error of the Mean = 0.04, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.18a - Malty Intensity: Treatment means after 0 or 5 days storage for each week.

Storage Time	Week	Treatment Means[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.2	0.0	0.1	0.2	0.1	0.4	0.2
	2	0.1	0.0	0.2	0.0	0.1	0.3	0.6
	4	0.1	0.0	0.2	0.4	0.0	0.2	0.1
	8	0.1	0.0	0.1	0.0	0.1	0.2	0.2
5 days	0	0.3	0.2	0.0	0.4	0.1	0.1	0.2
	2	0.1	0.0	0.2	0.1	0.4	0.2	0.0
	4	0.1	0.0	0.3	0.3	0.4	0.0	0.1
	8	0.0	0.0	0.2	0.0	0.0	0.1	0.0

[1] Standard Error of the Mean = 0.13, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.18b - Malty Intensity: Treatment means after 0 or 5 days storage independent of week.

Storage Time	Treatment Means by Storage Time[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.1	0.0	0.2	0.1	0.1	0.3	0.3
5 days	0.1	0.1	0.1	0.2	0.2	0.1	0.1

[1] Standard Error of the Mean = 0.06, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.18c - Malty Intensity: Treatment means for each week independent of storage time.

Week	Treatment Means by Week[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.2	0.1	0.0	0.3	0.1	0.2	0.2
2	0.1	0.0	0.2	0.0	0.2	0.3	0.3
4	0.1	0.0	0.2	0.3	0.2	0.1	0.1
8	0.0	0.0	0.2	0.0	0.1	0.1	0.1

[1] Standard Error of the Mean = 0.09, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.18d - Malty Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.1	0.0	0.1	0.2	0.1	0.2	0.2

[1] Standard Error of the Mean = 0.04, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.18e - Malty Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.2	0.2	0.2	0.1

[1] Standard Error of the Mean = 0.03 not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.18f - Malty Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.2
0	2	0.2
0	4	0.1
0	8	0.1
5	0	0.2
5	2	0.1
5	4	0.2
5	8	0.0

[1] Standard Error of the Mean = 0.05, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.19a - Feed Intensity: Treatment means after 0 or 5 days storage for each week.

Storage Time	Week	Treatment Means[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	2	0.0	0.0	0.0	0.1	0.1	0.0	0.0
	4	0.0	0.1	0.0	0.0	0.2	0.0	0.0
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5 days	0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.3	0.0	0.0	0.1	0.1	0.0
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.06, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.19b - Feed Intensity: Treatment means after 0 or 5 days storage independent of week.

Storage Time	Treatment Means by Storage Time[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.0	0.0	0.0	0.0	0.1	0.0	0.0
5 days	0.0	0.1	0.0	0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.03, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.19c - Feed Intensity: Treatment means for each week independent of storage time.

Week	Treatment Means by Week[1,2] CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.0^{a}	0.0^{a}	0.1^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
2	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
4	0.0^{b}	0.22^{a}	0.0^{b}	0.0^{b}	0.16^{ab}	0.0^{b}	0.0^{b}
8	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}

[1] Standard Error of the Mean = 0.04, significant, $p<0.01$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

[ab] Means within the same row sharing a common letter are not significantly different from each other , $p<0.05$

Table 4.19d - Feed Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.0	0.1	0.0	0.0	0.0	0.0	0.0

[1]Standard Error of the Mean = 0.03, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2]Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.19e - Feed Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.01^{ab}	0.01^{ab}	0.06^{a}	0.00^{b}

[1]Standard Error of the Mean = 0.02, significant, p<0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[ab] Means within the same row sharing a common letter are not significantly different from each other, p<0.05

Table 4.19f - Feed Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.0
0	2	0.0
0	4	0.0
0	8	0.0
5	0	0.0
5	2	0.0
5	4	0.1
5	8	0.0

[1]Standard Error of the Mean = 0.02, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.20a - Salty Intensity: Treatment means after 0 or 5 days storage for each week.

		Treatment Means[1,2]						
Storage Time	Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	2	0.0	0.0	0.0	0.1	0.0	0.1	0.0
	4	0.0	0.0	0.1	0.0	0.0	0.0	0.0
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5 days	0	0.1	0.1	0.0	0.1	0.0	0.0	0.0
	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.03, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.20b - Salty Intensity: Treatment means after 0 or 5 days storage independent of week.

	Treatment Means by Storage Time[1,2]						
Storage Time	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0 days	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5 days	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.01, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.20c - Salty Intensity: Treatment means for each week independent of storage time.

	Treatment Means by Week[1,2]						
Week	CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.02, not significant, p>0.05; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.20d - Salty Intensity: Treatment means independent of storage time and week.

Treatment Means[1,2]						
CTL	SAFF	SAFF/E	SAFF/M	SAFF/ME	FLAX/E	REF
0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.01, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[2] Diets were control (CTL), and control supplemented with, safflower oil (SAFF), safflower oil plus vitamin E (SAFF/E), safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME), or flaxseed plus vitamin E (FLAX/E); REF is the identified reference control

Table 4.20e - Salty Intensity: Mean values for each week independent of treatment or storage time.

Means by Week[1]			
0	2	4	8
0.0	0.0	0.0	0.0

[1] Standard Error of the Mean = 0.01, not significant, $p>0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

Table 4.20f - Salty Intensity: Mean values after 0 or 5 days storage for each week independent of treatment.

Storage Time	Week	Mean Score[1]
0	0	0.000[b]
0	2	0.020[ab]
0	4	0.009[ab]
0	8	0.000[b]
5	0	0.041[a]
5	2	0.000[b]
5	4	0.004[ab]
5	8	0.004[ab]

[1] Standard Error of the Mean = 0.02, significant, $p<0.05$; Scored from 0 to 4, 0=none, 4=extreme, see Appendix 1

[ab] Means within the same column sharing a common letter are not significantly different from each other, $p<0.05$

Table 4.21 - Milk fatty acid composition of treatment-pooled milk (treatments CTL, SAFF/M, and SAFF/ME from week 9) used for untrained consumer evaluation.

	Treatments[1]		
Fatty Acid[2]	CTL	SAFF/M	SAFF/ME
4:0	3.73	2.53	2.38
6:0	2.42	1.31	1.34
8:0	1.34	0.57	0.62
10:0	3.00	1.15	1.30
11:0	0.37	0.1	0.12
12:0	3.45	1.45	1.62
13:0	0.21	0.09	0.09
14:0	11.08	6.48	6.92
14:1	0.98	0.54	0.57
15:0	1.08	0.75	0.74
16:0	33.70	17.52	18.68
16:1	1.52	0.77	0.87
18:0	9.39	10.56	10.05
18:1 trans	2.33	20.94	19.10
18:1 n-9	16.53	18.11	18.76
18:1 n-7	0.40	0.62	0.58
18:2 t-11, c-15	0.39	0.65	0.54
18:2 n-6	1.62	2.91	2.84
18:3 n-3	0.38	0.33	0.30
20:0	0.19	0.16	0.16
20:1 n-12	0.16	0.12	0.14
20:1 n-9	0.07	0.12	0.11
CLA c-9, t-11	0.60	5.07	5.25
CLA t-10, c-12	0.01	0.09	0.07
Other FA	5.04	7.08	6.87

[1] Diets were control (CTL), and, control supplemented with: safflower oil plus monensin (SAFF/M) or safflower oil plus monensin plus vitamin E (SAFF/ME).

[2] Fatty acids are expressed as a percentage of fatty acid methyl esters

Table 4.22 – Flavor, mouthfeel, and overall acceptability of CTL, SAFF/M, and SAFF/ME milk as evaluated by an untrained consumer panel.

	Treatment[1,2]			
Sensory Attribute	CTL	SAFF/M	SAFF/ME	Sem[1]
Overall Acceptability	7.0^{a}	6.4^{b}	6.9^{a}	0.15*
Flavor	7.1^{a}	6.3^{b}	6.9^{a}	0.15***
Mouth Feel	7.0^{a}	6.7^{a}	7.1^{a}	0.15^{NS}

[1] *, *** Significant at $p<0.05$ and $p<0.001$, respectively; NS = Not significant; See Appendix 2 for scoring

[2] Diets were control (CTL), and control supplemented with, safflower oil plus monensin (SAFF/M), safflower oil plus monensin plus vitamin E (SAFF/ME)

[ab] Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$.

Table 4.23a - Comments from untrained consumer evaluation – characteristics panelists "liked" about samples

Comments:	CTL	SAFF/M	SAFF/ME
Taste/flavour	19	15	22
Tasted sweet	3	3	5
Not too sweet	1		
Tasted more like milk/good taste of milk	6	1	3
Flavour not too strong/lighter in taste	6	1	5
Creamy flavour/rich, full flavour/lots of flavour	4	3	2
Tastes natural like regular milk - 1%	1		
Tastes Bland			1
Smooth taste		1	1
Clean taste		1	1
Good aftertaste	2	1	
No milky aftertaste/limited aftertaste	3	2	1
No smell	1	1	1
Texture	2	5	3
Not too thick/creamy/heavy/rich	8	4	6
Creamy/thicker/creamy taste	5	1	5
Thickness	1	1	
Good consistency/smooth	4	4	4
Good mouth feel	1	2	4
Didn't leave film in mouth	2	1	2
Feels like milk	1		
Appearance	2	3	3
Colour	1	3	1
Colour - white/creamy	1	4	1
Rich colour - yellow white			1
Full, rich colour	1		
Looked creamy/thick	1	2	2
Didn't look too thick	1	1	
Looks inviting			1
Looks smooth	1		
Total Positive Comments:	**78**	**60**	**75**

Table 4.23b - Comments from untrained consumer evaluation – characteristics panelists "disliked" about samples

Comments:	CTL	SAFF/M	SAFF/ME
Taste/flavour	1	4	2
Thick tasting/rich flavour/too creamy/strong	3	4	7
Weak flavour/no taste	4	2	6
Sweet	1	3	3
Plastic/artificial/"different" taste		4	
Sour taste		2	
Tasted off (past best before date)		1	
Bitter taste		1	
Metallic taste		1	
Flat taste			1
Aftertaste/leaves taste in mouth	4	5	2
Not cold enough	1	2	2
Bit of a smell		1	
Watery/thin/not a real body to it/lack creamy	6	4	5
Too thick/heavy consistency	4	3	3
Left film in mouth	1	1	
Mouth feel		1	
Colour not pure white	1		
Light colour			1
Foamy/frothy/too many bubbles	4		1
Oil-like beads floating on surface	1		1
Looks like it was separating/powder mix	1		1
Looks watery	1		
Looked like had things floating on top	1		
Total Negative Comments:	**34**	**39**	**35**

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Chapter 5 - Enhancing the concentration of conjugated linoleic acid in bovine milk fat: Evaluation of the effect of synthetic conjugated linoleic acid on milk component secretion[1]

5.1 Introduction

Conjugated linoleic acid (CLA) refers to a group of 18-carbon conjugated dienoic acids of predominantly ruminant origin. Ruminant animals produce CLA through the biohydrogenation of dietary fat in the rumen and also by desaturation of trans-11 18:1 in the mammary gland and adipose tissue (Griinari et al., 2000). Conjugated linoleic acid can also be synthesized in the laboratory from vegetable oils like sunflower. Mixtures of CLA isomers have been reported to have anti-cancer, anti-obesity and anti-diabetic properties in various animal models (Belury, 2002).

Conjugated linoleic acid of ruminant origin contains predominantly one isomer (cis-9, trans-11 18:2) whereas synthetically produced CLA tends to contain a considerable proportion of trans-10, cis-12, as well as cis-9, trans-11 and smaller quantities of other isomers. This synthetic mixture of isomers has been shown to reduce subcutaneous fat and increase lean in pigs (Dugan et al., 1997) and will likely become commercially available in the near future. The trans-10, cis-12 isomer has been associated with these effects on body composition whereas the anticarcinogenic properties have been attributed to both the cis-9, trans-11 and trans-10, cis-12 isomers (Pariza et al., 2001). Isomers of CLA have also been found to have an inhibitory effect on bovine milk fat synthesis (Loor and Herbein, 1999). The trans-10, cis-12 CLA appears to be the isomer responsible for this effect (Baumgard et al., 2000). Rumen-protected CLA may therefore be beneficial as a tool to increase the protein to fat ratio in milk (Bauman and Griinari, 2001), and potentially improve the energy balance of early lactation cows (Perfield II et al., 2002).

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[1] A version of this chapter has been published in the Journal of Dairy Science, 2003, Vol. 86:1321-1324

In view of the ability of the trans-10, cis-12 isomer to reduce body fat in animals, interest has been shown in whether this isomer could have a benefit for weight reduction in humans. Feeding rumen-protected CLA could be a means of elevating the concentration of CLA isomers in bovine milk fat, thereby increasing the supply of these specific FA in the human diet. Gulati et al (2000) fed goats 40g/d of a rumen-protected CLA mixture for four days and observed a significant increase in milk fat CLA concentration. Similarly, Chouinard et al. (1999a) showed that post-ruminal delivery of 150g/d of a CLA mixture for five days was effective at increasing the concentration of CLA isomers in bovine milk fat. However, this level of CLA significantly depressed milk fat and reduced milk yield by more than 3 kg (Chouinard et al., 1999a). It was hypothesized that post-ruminal delivery of a mixture of CLA isomers for longer periods (that is, greater than five days) would have a significant negative impact on milk yield and composition. Our objective was to evaluate in greater detail the effect of post-ruminal delivery of CLA isomers on milk production and composition using abomasal infusion of 150g/d of a synthetic CLA mixture for 11-d periods.

5.2 Methods and Materials

5.2.1 Animals and Treatments

All procedures involving the use of animals were approved by the Faculty Animal Policy and Welfare Committee at the University of Alberta. Four multiparous pregnant Holstein cows were used in this study. The animals averaged 168 ± 21 DIM at the start of the trial with an average body weight (kg) of 675 ± 34 and an average body condition score (5-point scale) of 3.00 ± 0.29. All cows were fed the same basal diet of 50% forage (alfalfa silage, barley silage, and alfalfa hay) and 50% concentrate (based on barley, corn and canola meal) (Table 5.1). Each cow received abomasal infusion of: (1) control, no lipid infusion (CTL), (2) 150g/d of synthetic CLA (Conlinco Inc, Detroit Lakes, MN), 31.7% cis-9, trans-11; 30.4% trans-10, cis-12 (CLA), (3) 150g/d of safflower oil, 76% linoleic acid (SAFF), and (4) 150g/d of beef tallow (TALL). The lipids were infused daily in a 6-L carrier solution to give an adequate volume for infusion. The infusion solutions were made as follows. Briefly, 150g of lipid was made into a paste with 56g of spray-dried chicken protein (American Protein Corporation, Iowa) in a steam-jacketed kettle. Six liters of water were added and the mixture was stirred and heated. When the temperature reached 70°C, 7g of Tween-80 was added as an emulsifying agent. The emulsion was then homogenized in a one-stage 15M-8BA Gaulin homogenizer (Manton-Gaulin Manufacturing Co. Inc., Everett, MS, USA) at 2500 psi and stored at 4°C until needed. The infusate was pumped using a Masterflex® peristaltic pump with L/S-16 Tygon® tubing (Labcor Inc., Quebec, Canada). The tubing was passed through a rumen cannula and anchored in the abomasum with a plastisol flange. Infusion was carried out for 20-22 h/d for 11-d periods in a 4 x 4 Latin square design.

5.2.2 Sampling and Analysis

Dry matter intake and milk yield were recorded daily. Milk was sampled during the last two days of each period for composition analysis. Proximate analysis of feed, and determination of sodium and choride in milk, were carried out at Norwest laboratories (Leithbridge, AB). Microbial analysis of milk for *Streptocuccus/enterococcus* and *Staphylococcus aureus* was carried out at Norwest laboratories (Calgary, AB). Milk was analyzed for protein, fat, lactose, and somatic cell count (SCC) using near infrared spectroscopy at the Alberta Agriculture, Food and Rural Development Central Milk testing laboratory (Edmonton, AB). Fatty acid composition of milk was analyzed following preparation of FA methyl esters with sodium methoxide (Chouinard et al., 1999b).

Blood was sampled into heparin-containing tubes (Vacutainer, Becton Dickinson and Co., Rutherford, NJ) from the tail-vein after morning milking on the last day of each period. Blood was immediately centrifuged at 1500 x g for 15min to isolate the plasma. The plasma was removed, aliquoted, and stored frozen at -70°C until analysis. Plasma lipids were extracted using a modified Folch method (Folch et al., 1957) followed by separation of lipid fractions using thin-layer-chromatography (TLC) (Touchstone et al., 1979). Lipid fractions were methylated by sodium methoxide (Christie, 1982) and analyzed by gas chromatography as described previously (Chapter 3). The extraction, separation, and methylation procedures are described briefly as follows. Potassium choride (1.6ml; 0.1M) was added to the plasma sample. The mixture was transferred to a 9ml screw-cap tube and vortexed. To this tube were added (in succession) 0.8ml methanol, 2.0ml chloroform:methanol (1:1), 2.7ml chloroform, and 2.5ml chloroform:methanol (2:1), with vortexing between each addition. This was let stand overnight at 4°C. The bottom chloroform layer was then transferred to a 10ml disposable glass tube and dried down under nitrogen. Separation of the lipid fractions (triglycerides, cholesterol esters, phospholipids) was performed using a 10 x 10cm high performance TLC H-plate (Fisher Scientific, Ottawa, ON). The TLC filter plate was saturated for 30min prior to use in 15ml of solvent mixture (30ml choroform, 9ml methanol, 25ml 2-propanol, 6ml 0.25% w/w potassium chloride, 18ml triethlamine) contained in a 10 x 10cm tank. The lipid samples were reconstituted in 100µl of choroform:methanol (1:1) and applied to the plate using a 100µl Hamilton syringe. Another 50µl of choroform:methanol were added to the sample tube and applied to the same band. The plate was run until the solvent front reached the top of the plate (~ 45 to 60min). Samples were sprayed with 0.1% w/v 8-anilino-1-naphthalene sulfonic acid (ANSA) followed by visualization under UV. Each band was scored, scraped onto weighing paper, and transferred to a 15ml screw-cap tube for methylation. One milliliter of hexane was added to the sample followed by 2ml of 0.5M sodium methoxide. This was incubated at 50 °C for 15min. The reaction was terminated with 0.1ml glacial acetic acid. Five milliliters of double distilled water were added followed by 2ml of hexane. The tube was centrifuged at 1000rpm for 10min and the hexane layer extracted for gas chromatography analysis.

5.2.3 Statistical Analysis

The data was analyzed as a 4 x 4 Latin square design using the General Linear Models procedure of SAS (SAS, 1989). The model included the effect of cow, period, and treatment. Effect of carry-over was tested but found not significant and removed from the analysis. Treatment differences were evaluated using the Duncan procedure with a significance level of $P<0.05$.

5.3 Results and Discussion

The fatty acid composition of the lipids infused is shown in table 5.2. The CLA used was a two-isomer mixture made up of approximately equal amounts of the cis-9, trans-11 and trans-10, cis-12 isomers with small amounts of trans/trans and cis/cis isomers (Figure 5.1b). The important difference between this CLA and the 4-isomer mixture (Figure 5.1a) is the absence of cis/trans 11/13 and cis/trans 8/10 isomers. The infusion of safflower oil was a useful comparison to CLA in that it delivered a similar quantity of 18:2, but in a non-conjugated form. The tallow was made up of mainly 18:1 n-9, 18:0, and 16:0 and so delivered a mixture of fatty acids similar to the type of fatty acids that normally come from the rumen. In this way it differed from CLA and SAFF in being higher in saturated and monounsaturated fatty acids.

Infusion of CLA had dramatic effects on milk production and composition (Table 5.3). Milk yield declined steadily throughout the period of CLA infusion so that over the last two days of the period milk yield was 34.8 to 43.6% lower with CLA infusion compared to the other treatments. Concentration and yield of lactose and fat were also significantly lower with CLA infusion. Protein concentration was significantly higher with CLA although the yield of protein was lower compared to the other treatments. Analysis of fatty acid composition showed that the concentration of 18:2n-6 and conjugated linoleic acid increased significantly as a result of SAFF and CLA infusion, respectively (Table 5.4). Since the yield of milk fat was reduced with CLA infusion, the yield of all the fatty acids (except CLA isomers) was significantly reduced with the CLA treatment (Table 5.5).

The fatty acid composition of each of the main plasma lipid classes is presented in Table 5.6a and 5.6b. Although not measured in this study, a review by Christie (1978) reported bovine plasma lipids as consisting of 57% cholesterol esters, 4.8% triglycerides, 9.6% free cholesterol, 2.4% free fatty acids, and 24.8% phospholipids, with phosphatidylcholine representing as much as 70% of the phospholipids. More recent authors have reported similar results in lactating cows (Offer, et al, 1999; Loor and Herbein, 1998; Thivierge, et al 1998). The lipid classes in ruminant plasma each have a somewhat characteristic fatty acid composition (Christie, 1978). Triglycerides tend to have relatively high proportions of saturated fatty acids (especially C16:0 and C18:0) with lower levels of polyunsaturated fatty acids, whereas the cholesterol esters tend to have lower levels of saturated fatty acids and high levels of C18:2 n-6 in particular, and C18:3 n-3. The main fatty acids in phospholipids are generally C16:0, C18:0, C18:1 n-9 and C18:2 n-6. These general characteristics were also observed in each of the lipid classes in this study (Table 5.6a, 5.6b). The fatty acid composition of all the lipid classes largely reflected the fatty acid composition of the lipid infused. The level of cis-9, trans-11 CLA and trans-10, cis-12 CLA increased significantly in all lipid classes with infusion of the CLA mixture compared to the other treatments. Safflower oil (SAFF) infusion, compared to other treatments, significantly increased the proportion of C18:2 n-6 and significantly reduced the proportion of C18:1 n-9 in all lipid classes. The reason for the drop in C18:1 with SAFF is unclear but may suggest a preference for C18:2 esterification in the enterocyte over C18:1. This could represent a method of ensuring effective retention of scarce essential fatty acids. The n-6 and n-3 fatty acids of twenty or more carbons in length are desaturation/elongation products of C18:2 n-6 and C18:3 n-3 respectively (Cook, 1996). The C22:1 n-9 is an elongation product of C18:1 n-9. SAFF and CLA generally reduced the levels of these desaturation/elongation products compared to CTL and TALL, the effect being seen most clearly in the phospholipid fraction. This may have been due to a reduction in the desaturation and/or the elongation reactions. Both C18:2 n-6 and CLA have been associated with inhibition of Δ-6 desaturase (Chang, et al. 1992; Kinsella et al. 1990; Belury and Kempa-Steczko, 1997). Overall, the analysis of both plasma and milk fatty acid composition demonstrated very clearly the potential of post-ruminal delivery of lipids to change the fatty acid profile in ruminant tissues.

Most interesting was the effect of treatment on milk SCC (Table 5.3). The SCC was approximately five to seven times greater as a result of CLA infusion compared to the other treatments, which had values at levels considered normal for healthy cows. Somatic cell count is a count of white blood cells and sloughed off epithelial cells in milk. High counts are generally indicative of an infection in the udder. However, we believe that infection was not the cause of the high SCC observed with CLA infusion. The high SCC was observed in each cow but only during the period when that cow received CLA infusion. Milk from the period preceding or following the CLA period always had much lower counts. The cows also showed no physical signs that may have indicated an infection. For instance, there was no effect of treatment on dry matter intake (Table 4.3). Furthermore, there were no visible signs of mastitis during milking at any time in the course of the experiment. Bacterial analysis of the milk showed counts of

Streptocuccus/enterococcus well within the normal range for raw milk and no signs of *Staphylococcus aureus* (data not shown). The surprisingly low concentration of lactose with CLA was counterbalanced by a higher concentration of sodium (Table 5.3, figure 5.2). This is normally observed when there is a decrease in lactose and is a mechanism whereby osmotic balance is maintained. The concentration of chloride, which closely follows sodium, was also higher with CLA infusion (Table 5.3, figure 5.3). During the early stages of involution similar changes are seen in the mammary secretion as were observed with infusion of CLA. Although purely speculative, it is possible that infusion of relatively large amounts of these synthetic CLA isomers was initiating dry-off mechanisms in the udder. Further work will be necessary to explore this by examining the changes in expression of genes involved in the process of bovine mammary involution.

5.4 Conclusion

This study demonstrated that post-ruminal delivery of a mixture of CLA isomers could significantly increase the concentration of these various fatty acids in bovine milk. This is in contrast to methods that use modifications to the cow's diet to increase the natural production of CLA where the increase is predominantly in cis-9, trans-11 CLA. This study showed that the extent of enrichment possible for trans-10 isomers of CLA is limited because of unacceptable effects on milk yield and composition. This places a constraint on the degree to which synthetic CLA preparations could be used to enrich milk CLA and the extent to which bovine milk could be used as a vehicle to increase the supply of trans-10, cis-12 CLA in the human diet.

Table 5.1 - Ingredient and chemical composition of basal diet.

	% of DM
Ingredient composition	
Barley silage	20
Alfalfa silage	20
Alfalfa hay	10
Rolled barley	24.6
Ground corn	6.98
Canola meal	6.0
Corn gluten meal	2.18
Soybean meal	2.0
Fishmeal	1.9
Tallow	1.77
Corn distillers grain	1.2
Molasses	0.59
Megalac	0.45
Vitamin E[1]	0.45
Sodium bicarbonate	0.44
Biofos	0.36
Limestone	0.31
Magnesium oxide	0.27
Iodide salt	0.18
Mineral salt[2]	0.16
Vitamin ADE[3]	0.09
Vitamin D3[4]	0.03
Chemical composition	
DM, %	57.8
CP	15.9
Ether extract	4.5
NDF	38.9
ADF	24.4
Ash	7.53
NE_L[5], Mcal/Kg	1.61

[1] Contained: Vitamin E (5000 IU/kg)

[2] Contained: salt (min 95%), iodine (150 mg/kg), cobalt (50 mg/kg), copper (3500 mg/kg), manganese (10,000 mg/kg), zinc (9,000 mg/kg), and selenium (75 mg/kg).

[3] Contained: vitamin A (min 10,000,000 IU/kg), vitamin D (min 1,000,000 IU/kg), vitamin E (min 10,000 IU/kg).

[4] Contained: Vitamin D3 (5,000,000 IU/kg)

[5] Estimated from NRC (1989)

Table 5.2 - Fatty acid composition of lipid treatments

Fatty acid[1]	TALL[2]	SAFF	CLA
4:0-15:0	3.13	0.20	0.30
16:0	27.9	6.97	8.50
16:1 n-7	3.42	0.15	0.15
17:0	0.76	nd	nd
18:0	13.3	2.07	4.94
18:1 trans	1.34	nd	nd
18:1 n-9	41.8	14.2	19.2
18:2 n-6	6.82	76.1	0.96
Cis-9, trans-11 CLA	0.17	nd	31.7
Trans-10, cis-12 CLA	nd	nd	30.4

[1] Fatty acids expressed as percentage of fatty acid methyl esters.

[2] TALL = beef tallow; SAFF = safflower oil; CLA = synthetic conjugated linoleic acid.

nd = Not detected

Table 5.3 – Dry matter intake, milk yield, and milk composition from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), or conjugated linoleic acid (CLA).

	CTL[2]	TALL	SAFF	CLA	sem
Dry matter intake, kg/day[1]	18.59	18.92	19.27	18.59	0.56
Milk yield, kg/day	24.2^a	23.0^a	26.6^a	15.0^b	1.93
Lactose %	3.86^a	3.88^a	4.06^a	3.36^b	0.08
Lactose yield, kg/day	0.95^a	0.92^a	1.08^a	0.50^b	0.07
Fat %	2.38^a	2.62^a	2.37^a	1.78^b	0.16
Fat yield, kg/day	0.56^a	0.59^a	0.63^a	0.24^b	0.03
Protein %	3.06^a	2.99^a	3.16^a	4.42^b	0.20
Protein yield, kg/day	0.70^{ab}	0.66^a	0.83^b	0.61^a	0.04
Somatic cell count, x1000/ml	187^a	193^a	133^a	991^b	144.30
Sodium, mg/kg	715^a	748^a	658^a	978^b	64.40
Chloride, %	0.192^a	0.192^a	0.188^a	0.252^b	0.01

[a,b,c] Within a row, values with different superscripts are significantly different ($P < 0.05$).

[1] Values for dry matter intake represent average for entire period since dry matter intake remained stable during each period regardless of treatment; milk parameters represent values for the last two days/period.

[2] CTL is control (no lipid infusion); TALL is infusion of 150g/day beef tallow; SAFF is infusion of 150g/day of safflower oil; CLA is infusion of 150g/day of synthetic CLA.

Table 5.4 - Fatty acid composition of milk fat from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), or conjugated linoleic acid (CLA).

Fatty acid[1]	CTL[2]	TALL	SAFF	CLA	sem
4:0-15:0	23.6^{a}	22.3^{a}	21.9^{a}	18.2^{b}	0.91
16:0	32.3^{a}	31.9^{a}	29.9^{a}	39.5^{b}	1.12
16:1 n-7	1.41^{ab}	1.58^{a}	1.12^{b}	1.39^{ab}	0.12
17:0	0.62	0.58	0.57	0.62	0.02
18:0	11.0^{a}	11.0^{a}	11.2^{a}	13.5^{a}	0.80
18:1 trans	2.48	2.70	2.58	2.21	0.16
18:1 n-9	24.4^{ab}	25.6^{a}	22.8^{b}	18.2^{c}	0.70
18:1 n-12	1.13	1.08	1.09	0.96	0.08
18:2 n-6	1.8^{a}	2.1^{a}	7.6^{b}	2.3^{a}	0.20
18:3 n-3	0.15	0.15	0.17	0.19	0.02
Cis-9, trans-11 CLA	0.59^{a}	0.61^{a}	0.58^{a}	1.77^{b}	0.11
Trans-10, cis-12 CLA	ND^{a}	ND^{a}	ND^{a}	0.85^{b}	0.06

[a,b,c] Within a row, values with different superscripts are significantly different ($P < 0.05$).

[1] Fatty acids expressed as percentage of fatty acid methyl esters.

[2] CTL is control (no fat infusion); TALL is infusion of 150g/day beef tallow; SAFF is infusion of 150g/day of safflower oil; CLA is infusion of 150g/day of synthetic CLA.

ND = Not detected

Table 5.5 - Yield of fatty acids in milk fat from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), or conjugated linoleic acid (CLA).

Fatty acid (g/day)	CTL[1]	TALL	SAFF	CLA	sem
C4-15	124^{a}	120^{a}	136^{a}	39^{b}	11.46
C16:0	172^{a}	176^{a}	186^{a}	81^{b}	11.98
C16:1 n-7	7.7^{a}	8.7^{a}	6.9^{a}	2.8^{b}	0.63
C17:0	3.3^{a}	3.2^{a}	3.6^{a}	1.3^{b}	0.24
C18:0	58.5^{a}	60.3^{a}	70.5^{a}	29.8^{b}	5.28
C18:1 trans	12.9^{b}	14.4^{ab}	15.9^{a}	5.0^{c}	0.67
C18:1 n-9	130^{a}	137^{a}	142^{a}	38.7^{b}	9.54
C18:1 n-12	5.8^{a}	5.8^{a}	6.8^{a}	2.2^{b}	0.37
C18:2 n-6	9.5^{a}	10.9^{a}	46.8^{b}	4.7^{c}	0.86
C18:3 n-3	0.94^{ab}	1.0^{a}	1.1^{a}	0.41^{b}	0.16
Cis-9, trans-11 CLA	3.08^{a}	3.31^{a}	3.59^{a}	3.90^{a}	0.43
Trans-10, cis-12 CLA	0^{a}	0^{a}	0^{a}	1.86^{b}	0.27

[a,b,c] Within a row, values with different superscripts are significantly different ($P < 0.05$).

[1] CTL is control (no fat infusion); TALL is infusion of 150g/day beef tallow; SAFF is infusion of 150g/day of safflower oil; CLA is infusion of 150g/day of synthetic CLA.

Table 5.6a - Fatty acid composition of plasma total phospholipids and phosphatidylcholine from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), or conjugated linoleic acid (CLA)

	Total Phospholipids					Phosphatidylcholine				
Fatty acid[1]	CTL[2]	TALL	SAFF	CLA	sem	CTL	TALL	SAFF	CLA	sem
C12:0	nd	nd	nd	nd	-	nd	nd	nd	nd	-
C14:0	0.12	0.14	0.17	0.14	0.058	0.12^{a}	0.14^{b}	0.12^{a}	0.14^{b}	0.004
C14:1	0.77^{a}	1.28^{b}	0.86^{a}	0.82^{a}	0.059	nd	nd	nd	nd	-
C15:0	0.54	0.58	0.75	0.62	0.032	0.41^{ab}	0.40^{ab}	0.44^{a}	0.37^{b}	0.01
C16:0	14.7	14.7	15.0	15.3	0.170	14.8	14.8	14.8	15.4	0.21
C16:1	0.38^{a}	0.40^{a}	0.22^{b}	0.28^{b}	0.015	0.40^{a}	0.44^{a}	0.22^{b}	0.33^{c}	0.02
C17:0	1.04^{a}	1.02^{a}	1.17^{b}	0.95^{a}	0.016	1.12^{a}	1.06^{a}	1.12^{a}	0.95^{b}	0.03
C18:0	23.7^{ab}	22.6^{a}	22.9^{ab}	24.0^{b}	0.159	23.7^{a}	22.8^{b}	22.8^{b}	24.1^{a}	0.22
C18:1 trans	1.00^{ab}	1.17^{b}	0.88^{a}	0.92^{a}	0.031	0.99^{ab}	1.17^{b}	0.93^{a}	0.97^{ab}	0.06
C18:1 cis	12.2^{a}	12.7^{a}	5.0^{b}	13.1^{a}	0.304	13.2^{a}	13.3^{a}	5.6^{b}	13.4^{a}	0.36
C18:2	29.5^{a}	29.8^{a}	42.7^{b}	28.4^{a}	0.244	30.2^{a}	31.6^{a}	43.6^{b}	29.4^{a}	0.59
C18:3 (n-6)	0.25^{a}	0.21^{ab}	0.10^{b}	0.16^{ab}	0.008	0.22^{a}	0.19^{a}	0.09^{b}	0.12^{b}	0.02
C18:3 (n-3)	1.88^{a}	1.67^{a}	0.54^{b}	1.87^{a}	0.044	1.92^{a}	1.67^{a}	0.67^{b}	1.88^{a}	0.10
CLA (c9/t11)	0.06^{a}	0.12^{a}	nd^{a}	1.34^{b}	0.062	0.17^{a}	0.15^{a}	0.10^{a}	1.43^{b}	0.12
CLA (t10/c12)	nd^{a}	nd^{a}	nd^{a}	1.14^{b}	0.052	nd^{a}	nd^{a}	nd^{a}	0.94^{b}	0.08
C20:2 (n-6)	0.21^{a}	0.25^{a}	0.14^{b}	0.15^{b}	0.006	0.19^{a}	0.20^{a}	0.14^{b}	0.14^{b}	0.01
C20:3 (n-6)	nd	nd	nd	nd	-	nd	nd	nd	nd	-
C20:4 (n-6)	2.42^{a}	2.04^{a}	1.48^{b}	1.53^{b}	0.064	2.28^{a}	2.01^{a}	1.43^{b}	1.50^{b}	0.13
C20:5	0.90^{a}	0.78^{a}	0.44^{b}	0.76^{a}	0.021	0.86^{a}	0.78^{ac}	0.49^{b}	0.72^{c}	0.03
C22:1 (n-9)	2.34^{a}	2.24^{a}	1.68^{b}	1.87^{b}	0.031	2.12^{a}	2.19^{a}	1.48^{b}	1.78^{b}	0.09
C22:4 (n-6)	0.19^{a}	0.18^{a}	0.14^{b}	0.17^{c}	0.002	0.17^{a}	0.17^{a}	0.13^{b}	0.15^{ab}	0.01
C22:5	0.87^{a}	0.86^{ab}	0.74^{b}	0.78^{ab}	0.015	0.78	0.90	0.81	0.82	0.07
C22:6 (n-3)	1.37^{a}	1.37^{a}	1.11^{b}	1.29^{a}	0.019	1.13^{ab}	1.18^{b}	0.96^{c}	1.08^{a}	0.02
Other	5.57	5.96	4.11	4.42	0.259	5.20^{a}	4.84^{ab}	4.06^{c}	4.24^{bc}	0.18

^{a,b,c}Within a row, values with different superscripts are significantly different ($P < 0.05$).

1 Fatty acids expressed as percentage of fatty acid methyl esters.

2 CTL is control (no fat infusion); TALL is infusion of 150g/day beef tallow; SAFF is infusion of 150g/day of safflower oil; CLA is infusion of 150g/day of synthetic CLA.

ND = Not detected

Table 5.6b - Fatty acid composition of plasma cholesterol esters and triglycerides from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), or conjugated linoleic acid (CLA)

	Cholesterol esters					Triglycerides				
Fatty acid[1]	CTL[2]	TALL	SAFF	CLA	sem	CTL	TALL	SAFF	CLA	sem
C12:0	0.13	0.13	0.14	0.13	0.015	1.30	0.70	0.63	0.85	0.08
C14:0	1.06^{ab}	1.01^{a}	0.86^{c}	1.14^{b}	0.029	2.21	1.97	1.98	2.10	0.08
C14:1	nd	nd	nd	nd	-	nd	nd	nd	nd	-
C15:0	0.62	0.58	0.44	0.52	0.064	1.18	1.03	1.10	1.08	0.05
C16:0	3.78^{a}	3.74^{a}	3.32^{b}	3.69^{a}	0.056	30.87	30.16	29.41	31.95	0.86
C16:1	1.61^{a}	1.66^{a}	0.88^{b}	1.42^{a}	0.048	1.06	1.63	0.89	0.81	0.06
C17:0	0.51	0.60	0.34	0.53	0.103	1.21	1.23	1.18	1.25	0.04
C18:0	0.36^{ab}	0.36^{ab}	0.34^{a}	0.40^{b}	0.015	34.28	33.30	35.08	31.94	0.43
C18:1 trans	0.12	0.16	0.12	0.12	0.014	5.84	6.14	5.60	4.99	0.12
C18:1 cis	3.60^{a}	3.42^{a}	2.30^{b}	3.44^{a}	0.208	10.86	10.67	8.62	9.21	0.69
C18:2	67.6^{a}	68.3^{a}	75.5^{b}	66.6^{a}	1.291	3.65	3.08	7.20	4.58	0.59
C18:3 (n-6)	1.45^{a}	1.10^{b}	0.71^{c}	0.90^{bc}	0.091	0.43	0.48	0.65	0.70	0.11
C18:3 (n-3)	8.32^{a}	7.44^{a}	4.58^{b}	8.46^{a}	0.478	1.24	1.37	0.97	1.20	0.11
CLA (c9/t11)	0.12^{a}	0.11^{a}	0.15^{a}	0.54^{b}	0.028	nd^{a}	0.29^{a}	nd^{a}	2.07^{b}	0.15
CLA (t10/c12)	nd^{a}	nd^{a}	nd^{a}	0.39^{b}	0.007	nd^{a}	nd^{a}	nd^{a}	1.93^{b}	0.03
C20:2 (n-6)	0.02^{a}	nd^{a}	0.09^{a}	0.26^{b}	0.028	nd	nd	nd	nd	-
C20:3 (n-6)	nd	nd	nd	nd	-	nd	nd	nd	nd	-
C20:4 (n-6)	0.32^{a}	0.28^{ab}	0.22^{b}	0.27^{ab}	0.020	nd	nd	nd	nd	-
C20:5	1.22^{a}	1.14^{ab}	0.87^{b}	1.16^{ab}	0.100	0.48	0.44	0.35	0.29	0.10
C22:1 (n-9)	1.28^{a}	1.21^{a}	0.97^{b}	1.16^{ab}	0.060	0.06	0.11	0.12	0.08	0.03
C22:4 (n-6)	0.24	0.16	0.15	0.14	0.066	nd	nd	nd	nd	-
C22:5	nd	nd	nd	nd	-	0.08	0.21	0.05	0.07	0.03
C22:6 (n-3)	0.11	0.16	0.03	0.04	0.040	1.53	1.57	1.48	1.12	0.10
Other	7.81	8.41	7.94	8.61	0.716	5.33	5.32	4.70	5.02	0.11

[a,b,c] Within a row, values with different superscripts are significantly different ($P < 0.05$).

[1] Fatty acids expressed as percentage of fatty acid methyl esters.

[2] CTL is control (no fat infusion); TALL is infusion of 150g/day beef tallow; SAFF is infusion of 150g/day of safflower oil; CLA is infusion of 150g/day of synthetic CLA.

ND = Not detected

Figure 5.1 - Ag$^+$ High Performance Liquid Chromatography analysis of (a) 4 isomer CLA oil and (b) 2-isomer CLA oil

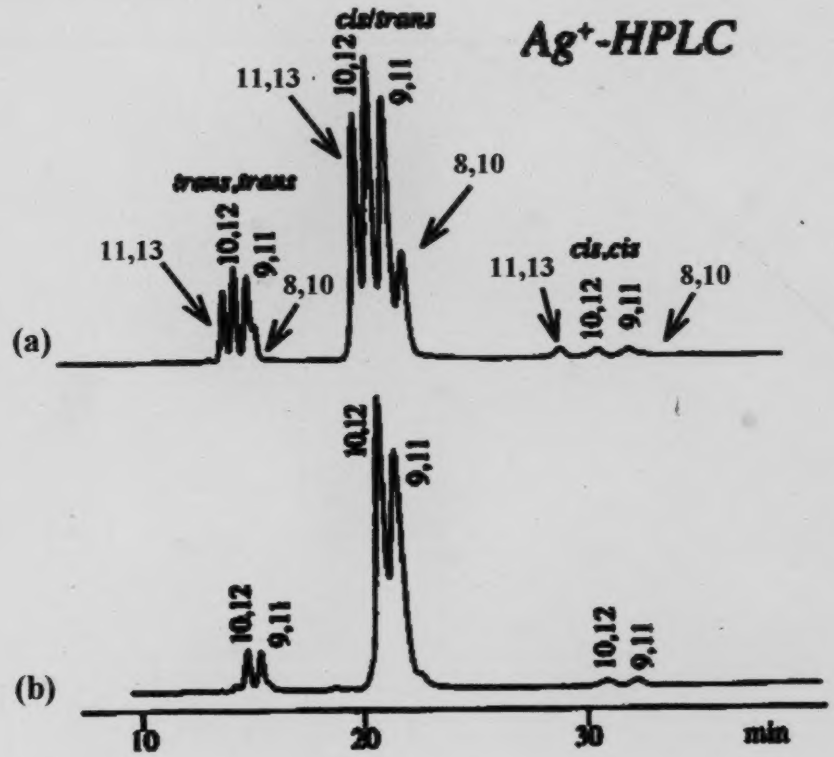


Figure 5.2 – Relationship between the concentration of lactose and sodium in milk from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), and conjugated linoleic acid (CLA).

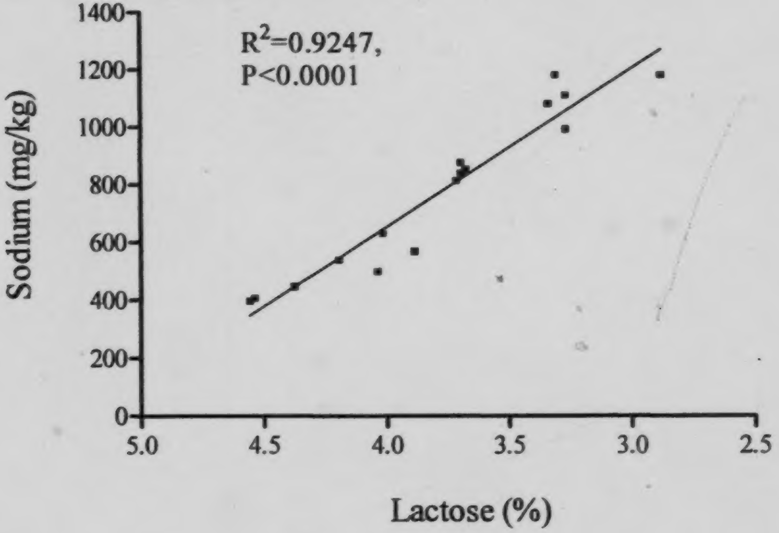
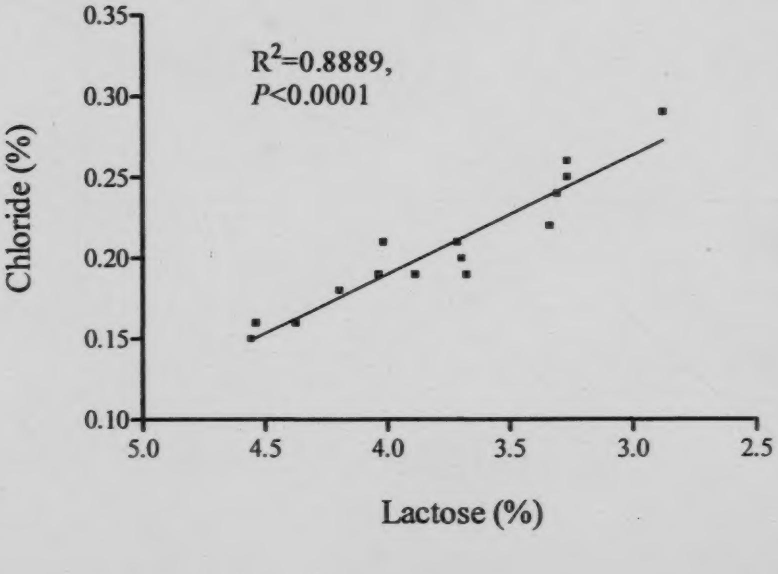


Figure 5.3 – Relationship between the concentration of lactose and choride in milk from cows receiving abomasal infusion of control (CTL), beef tallow (TALL), safflower oil (SAFF), and conjugated linoleic acid (CLA).



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Chapter 6 – General Discussion and Conclusions

6.1 Introduction

Although bovine milk fat is composed almost entirely of triglycerides, these triglycerides contain an extremely diverse range of fatty acids (Jensen, 2002). Milk fatty acids can be four to twenty-four carbons in length, and include saturated, unsaturated, odd chain, branched chain, and trans fatty acids. Bovine milk fat has often been criticized for having a relatively high proportion of saturated fatty acids and a low proportion of polyunsaturated fatty acids (Berner, 1993). Particularly problematic has been the high concentration of 14:0 and 16:0. These fatty acids have been shown to increase blood low-density-lipoprotein cholesterol in some human studies, a major risk factor for coronary heart disease.

Researchers have been interested in modifying the composition of milk fat for decades. Much of this work has focused on feeding various sources of dietary lipid for the purpose of increasing the level of particular fatty acids: most often n-3 and n-6 polyunsaturated fatty acids. More recently, particular emphasis has been given to finding feeding regimens that will increase the concentration of conjugated linoleic acid (CLA) in ruminant milk fat. Despite the large volume of knowledge that has accumulated on how to modify the composition of cow's milk, very little of this information has been applied in a commercial setting. Yet, the ability to modify the composition of milk fat represents an opportunity for the development of novel dairy products such as CLA-enriched milk.

The discovery of the anticarcinogenic properties of CLA presented exciting new possibilities for the production of dairy products with modified fat content. The interesting aspect that makes increasing CLA in milk so different from increasing other types of unsaturated fatty acids is that while previous work struggled with the problem of avoiding the effects of rumen biohydrogenation, increasing CLA is dependent on the biohydrogenation process. The primary goal of the research presented in this thesis was to evaluate dairy feeding strategies aimed at increasing the concentration of CLA in bovine milk.

6.2 Enhancing the Concentration of Conjugated Linoleic Acid in Bovine Milk Fat

The presence of CLA in bovine milk is an intriguing outcome of the symbiotic relationship between the ruminant animal and the microorganisms it hosts. Of central importance to the production of CLA in the cow is the ability of rumen bacteria to hydrogenate unsaturated fatty acids originating from the diet. Rumen biohydrogenation was traditionally seen as an undesirable process, responsible for the high proportion of saturated fatty acids in bovine milk fat. Ironically, this same process produces isomers CLA and trans 18:1 fatty acids as intermediates. The CLA found in milk originates directly from the rumen as well as through mammary desaturation of trans-11 18:1 by stearoyl-CoA desaturase (Griinari and Bauman, 1999).

The studies presented in chapters 2 and 3 were based on the hypothesis that CLA in milk could be enhanced through manipulation of the biohydrogenation process. Previous research demonstrated that 18:2 n-6 is hydrogenated in the rumen to 18:0, producing CLA and trans-11 18:1 as intermediates, and that 18:3 n-3 goes through a similar pathway, producing trans-11 18:1 but not CLA (Kepler and Tove, 1967; Griinari and Bauman, 1999). The first and most obvious approach seemed to be to increase the amount of 18:2 n-6 and/or 18:3 n-3 in the cow's diet. In Alberta where the dairy diet is based on feeding conserved forages and grains, the most suitable source of 18:2 n-6 and 18:3 n-3 is oilseeds such as sunflower, soybean, safflower, flaxseed, and canola. Two oilseeds, safflower oil and flaxseed oil, were tested in the research presented in Chapters 2 and 3. Safflower was chosen for its very high content of 18:2 n-6 (76%) and flaxseed oil for its high content of 18:3 n-3 (41.7%). Feeding safflower or flaxseed oil at 6% of dietary DM was found to significantly increase the concentration of CLA in bovine milk fat, although safflower oil was more effective than flaxseed (Chapter 3, Table 3.6). The reason for this could be that in the rumen 18:2 n-6 is converted to CLA and trans-11 18:1 whereas 18:3 n-3 produces trans-11 18:1 but no CLA. Another factor is possibly that the 18:3 n-3 did not result is as much trans-11 18:1 production as might be predicted. With flaxseed feeding there was a substantial increase in the milk concentration of trans-11, cis-15 18:2, the direct precursor of trans-11 18:1 in the 18:3 n-3 biohydrogenation pathway. The accumulation of this intermediate would decrease the amount of trans-11 18:1 available for CLA synthesis in the rumen.

It was also hypothesized that dietary ingredients that interfered with the biohydrogenation process in a way that increased the accumulation of CLA and/or trans-11 18:1 would increase milk CLA concentrations. If the hydrogenation process could be manipulated to inhibit the conversion of CLA to trans-11 18:1 or trans-11 to 18:0 it should result in an increase of CLA in milk. Furthermore, modifying the biohydrogenation process to minimize pathways that produce trans-10 18:1 might also be expected to increase milk CLA. The biohydrogenation process appears to require the participation of a number of bacterial types, although knowledge in this area is still fairly limited. Antibiotic ionophores, which inhibit the growth of gram positive bacteria, have been shown *in vitro* to inhibit 18:2 n-6 biohydrogenation, resulting in an accumulation of trans-11 18:1 and CLA (Fellner et al., 1997). In the studies presented in this thesis (Chapters 2 and 3), the effect of the ionophore monensin was tested either alone or in combination with safflower oil for its ability to increase milk CLA. Feeding monensin in combination with safflower oil increased CLA and trans 18:1 in milk more than safflower alone. Including monensin in the diet without safflower oil did not increase milk CLA or trans 18:1 above the control (Chapter 2). These results showed that monensin, when fed with a source of 18:2 n-6, was an effective tool for increasing CLA concentrations in milk. An important concern with using monensin for this purpose is the public perception about antibiotic use in animal production. This could be an especially important issue if marketing CLA-enriched milk for its potential health promoting attributes. Although using monensin in dairy feeding does not present any health risks to humans consuming the milk, and is not equivalent to any antibiotics used in human medicine, the potential risk monensin might have for the image of CLA-enriched products might outweigh the benefits.

The study presented in Chapter 3 also tested the effect of vitamin E on milk CLA concentrations. Vitamin E had been shown to prevent some of the depression in milk fat caused by feeding oils high in polyunsaturated fatty acids (Charmley and Nicholson, 1993; Charmley and Nicholson, 1994; Focant et al., 1998). Current theory proposes that milk fat depression is caused by rumen-derived trans-10 fatty acids (Bauman and Griinari, 2001). We hypothesized that if vitamin E prevented milk fat depression by shifting biohydrogenation pathways away from trans-10 towards trans-11, it might also increase milk CLA by providing more trans-11 18:1 for mammary CLA synthesis. The study presented in Chapter 3 confirmed previous results from other laboratories that dietary vitamin E could partially prevent milk fat depression. However, the effect of vitamin E on CLA was less clear. The concentration of CLA was not significantly different for SAFF verses SAFF/E, or SAFF/M verses SAFF/ME. It is possible that the vitamin E effect on milk fat percent was brought about by an overall reduction in total trans 18:1. Vitamin E significantly reduced the concentration of total trans 18:1 in milk for SAFF/ME compared to SAFF/M, and non-significantly for SAFF/E compared to SAFF (Chapter 3, Table 3.6). Further analysis will be required to determine if the decrease in total trans 18:1 in milk was due to a reduction in trans-11, trans-10, or both.

The studies presented in Chapter 2 and 3 provided proof of concept that the concentration of CLA in bovine milk could be increased ten-fold through modification of the dairy diet. Further work will be necessary determine if the production of CLA-enriched milk is economically viable using the diets described in this thesis. It will also be useful to test cheaper sources of 18:2 n-6 and 18:3 n-3. Two potential candidates are sunflower and the Canadian developed Linola, a flaxseed variety high in 18:2 n-6 (71%). Another important direction for future work will be in gaining a greater understanding of the bacterial populations involved in rumen biohydrogenation in order to understand how this process can be better manipulated to increase milk CLA concentrations. Methods that prevented the conversion of CLA to trans 18:1 in the rumen might be advantageous in that they would increase milk CLA, without the large increase in trans 18:1.

The study presented in Chapter 5 evaluated the potential for post-ruminal delivery of synthetic CLA as a means to increasing CLA in bovine milk fat. Protecting dietary lipids from rumen biohydrogenation is a technique that increases the duodenal flow of polyunsaturated fatty acids (Ashes et al., 1997). Although protection is often less than complete, rumen-protected lipid sources are an effective method of changing the fatty acid composition of bovine milk. Feeding protected CLA could be particularly useful in situations where natural synthesis of CLA decreases, (for instance in the winter months of pasture fed cows), thereby maintaining a high CLA concentration year round. A limited number of studies have evaluated protected CLA, either as a method of increasing CLA in milk (Gulati et al., 2000), or as a method of decreasing milk fat percent (Bauman and Griinari, 2001). The study presented in Chapter 5 used abomasal infusion to simulate rumen-protected CLA. Most synthetic CLA sources available have been composed of a mixture of cis-9, trans-11 and trans-10, cis-12, along with a variety of other minor CLA isomers. The trans-10, cis-12 isomer, which is found normally only in minor quantities in milk, causes a sharp decline in milk fat synthesis even at low concentrations (Loor and Herbein, 1999). However, if protected synthetic CLA were to be used as a means of increasing CLA in milk, fairly large quantities of CLA would have to be fed over long periods of time. The effect of long-term post-ruminal delivery of large amounts of trans-10 isomers of CLA on milk synthesis was uncertain. The study presented in Chapter 5 showed that post-ruminal delivery of 150g/d of a mixture of CLA isomers had extremely negative effects on milk yield and composition (Chapter 5, Tables 5.3-5.5). This strongly suggested that the extent of enrichment possible for trans-10 isomers of CLA would be limited. As methods of CLA synthesis and purification become more refined and cheaper it may be possible to feed protected CLA that is composed of only cis-9, trans-11. The usefulness of protected CLA will depend on how effective the protection methods are, as well as the costs involved. A study by Campbell et al. (2003) took a different approach to increasing milk CLA by fortifying the milk with a synthetic mixture of CLA. However, the resulting product appeared to have less than satisfactory sensory characteristics. It would seem that the most practical approach to increasing CLA in bovine milk fat are methods that encourage the natural synthesis and incorporation of CLA into milk fat.

6.3 Producing CLA-enriched Dairy Products

The studies presented in Chapters 2 and 3 demonstrated the feasibility of producing milk with enhanced levels of CLA. The next step was an evaluation of the organoleptic characteristics of this milk (Chapter 4). CLA-enriched milk was collected at various time points over a period of two months, processed, and evaluated by a trained sensory panel. Overall, the CLA-enriched milk was not different from control milk in any of its sensory characteristics. This suggested that CLA-enriched milk would be an equally acceptable product for fluid milk consumption as the control, which represented "typical" Alberta milk. Further work will be required to evaluate the suitability of CLA-enriched milk for processing into dairy products such as butter and cheese. Although the changes in the fatty acid composition will undoubtedly necessitate changes in processing conditions to achieve an acceptable product, these problems are not likely to be insurmountable.

A greater challenge may be to convince the processing sector to invest in new product development. The long-term viability of the dairy industry depends on producing products to meet changing consumer demand. Consumers are becoming more conscious of the health attributes of the food they consume. CLA enriched milk may be attractive to those who have abandoned milk and milk products, such as butter, due to concerns over the impact of milk fat on their health. However, the introduction of new products like CLA enriched milk does require significant investment in marketing and there are no guarantees that the product will attract sufficient consumer interest to be viable. The incentive for producers to feed the special supplement needed to enhance CLA levels would be the payment of a premium price for the milk.

6.4 Assessing the Potential for CLA-Enriched Dairy Products

Consumers could increase their CLA intake by taking synthetic CLA in capsule form, which is already available in health food stores. The main difference between the CLA in these products and milk CLA is the broader range of isomers in the synthetically produced CLA. The relative value for human health of long-term consumption of this range of CLA isomers compared to the CLA found in ruminant milk fat is uncertain. The CLA produced through manipulation of the dairy ration has a market advantage over the synthetic product in that it can be promoted as a “natural” source of CLA. It may also be easier for CLA-enriched milk to gain acceptance since milk already has a wide distribution and consumers are well accustomed to seeing a broad variety of dairy products in the shops.

The challenge will be in overcoming the existing public perception regarding milk fat and health. The fact that CLA is a trans fatty acid may add to the confusion. This will be especially true as laws change to require the declaration of trans fatty acid content on food labels, and health experts advise the public to decrease their intake of trans fatty acids. An important area for future research will be on gaining a greater understanding of the biological activity of the trans 18:1 fatty acids in bovine milk fat. Two studies have carried out consumer surveys to assess the potential interest in CLA-enriched dairy products (Campbell et al., 2003; Ramaswamy et al., 2003). The study by Campbell et al. (2003) reported substantial interest among consumers for foods with "functional" properties, as well as a willingness to pay more for them. The study by Ramaswamy et al. (2003) concluded that consumers generally indicated enthusiasm for CLA-enriched products. One of the central questions the Ramaswamy et al. (2003) study asked was "Would you purchase a milk that prevents cancer?" Not surprisingly, the vast majority of respondents responded "yes". However, the usefulness of this predictable enthusiasm is limited since definitive proof that CLA prevents cancer in humans may prove difficult to achieve.

6.5 Concluding Remarks

The concept of enhancing the levels of health promoting fatty acids in food is not new. A good example of this has been the introduction of eggs enriched in omega-3 fatty acids. This recognizes the trend among consumers towards an increased desire to make diet choices that promote good health.

The potent health promoting effects of CLA has been an unanticipated discovery. Moreover, immense potential exists to increase the concentration of CLA in bovine milk fat using nutrition. The ability to enrich the level of CLA in dairy products could be useful for increasing CLA intake in humans, without the need for dramatic changes in food consumption patterns. It may also provide exciting new market opportunities for milk and milk products such as butter and cheese.

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Appendix 1

Trained Sensory Evaluation

Fluid Milk Testing Instructions

&

Evaluation Form

Trained Panel Testing Procedure

- Swirl the milk sample 5 times.
- Bring the sample vial to the nose, remove the lid and take 2 - 3 short sniffs.
- Evaluate the odor.
- Take the sample into the mouth and move it around making sure to coat all the surfaces of the mouth from the front to deep in the back of the throat, noting any off flavors.
- While the sample is in the mouth air is moved up through the nose to enhance odor detection.
- Expectorate the sample.
- Wait a few minutes and observe the aftertaste.
- Aftertaste and aroma are enhanced by exhaling slowly through the nose.
- Rinse with water, expectorate, eat a piece of cracker and rinse again, wait 1 minute before proceeding to the next sample.

DATE: NAME:

FLUID MILK EVALUATION

CODE:

ODOR

Overall Intensity C

Bland ________________________________ Extremely Intense

Off Odor Intensity

C

None ________________________________ Extreme

FLAVOR

Overall Intensity C

Bland ________________________________ Extremely Intense

Off Flavor Intensity

C

None ________________________________ Extreme

Sweet C

None ________________________________ Extreme

Aftertaste C

None ________________________________ Extreme

Evaluate the following off flavor notes using a scale of 0-4 where:

0= none **1= slight** **2= moderate** **3= very** **4= extreme**

FLAT _____
OXIDIZED _____
ACID/SOUR _____
RANCID/BITTER _____
MALTY _____
FEED _____
FRUITY _____
SALTY _____
ASTRINGENT _____
COOKED _____
COWY(ACETONE) _____
FOREIGN _____

OTHERS:

Descriptor	Score	Descriptor	Score
______________	_____	______________	_____
______________	_____	______________	_____

Mouth Feel

C

Thin ________________________________ Thick

Appendix 2

Untrained Consumer Evaluation

Fluid Milk Testing Instructions

&

Evaluation Form

EVALUATION PROCEDURES

DO NOT TALK WITH OTHER PANELISTS DURING THE EVALUATION.

MILK

- **BEFORE YOU START AND BETWEEN SAMPLES, take a bite of cracker and a drink of water.**
- **Check that the three digit code on the sample and the ballot are the same.**
- **Look at and taste the first sample. Answer all the questions about the sample in the order the questions are presented. Retaste as required.**
- **Proceed to evaluate the next samples, using the same procedures.**
- **Complete demographic questions.**

- **Please deposit contents of your tray in the garbage container at the back of the room and leave your tray on the counter.**
- **Check that you have answered all the questions, bring the ballot to reception.**

- **Please do not discuss the ballot with other potential panelists.**

THANK YOU FOR YOUR ASSISTANCE!!

☺

Have a great day!

Respondent # ________
Respondent Initials _______
Date: April 5, 2000
CODE: 996

MILK

Please take a bite of the cracker and cleanse the palate with water before you start and in between samples. Look at and taste the MILK SAMPLE. All things considered, indicate how much you liked/disliked it. Place a check in one of the boxes.

OVERALL ACCEPTABILITY

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like/Dislik	Like Slightly	Like Moderately	Like Very Much	Like Extremely

Please tell us what you liked or disliked about the milk sample, if anything.

LIKED	DISLIKED

PLEASE TURN PAGE

CODE: 996

Retaste as required and answer the following questions about the product.

FLAVOUR

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like/Dislik	Like Slightly	Like Moderately	Like Very Much	Like Extremely

MOUTH FEEL

☐	☐	☐	☐	☐	☐	☐	☐	☐
Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like/Dislik	Like Slightly	Like Moderately	Like Very Much	Like Extremely

PLEASE PROVIDE THE FOLLOWING DEMOGRAPHIC INFORMATION.

Gender: Male ________ Female: ________

Age: 18 - 24 ☐ 25 - 34 ☐ 35 - 44 ☐ 45 - 54 ☐ 55 - 64 ☐ 64+ ☐

THANK YOU
PLEASE PROCEED TO RECEPTION